



**AGRICULTURAL RESEARCH INSTITUTE  
PUSA**





# THE JOURNAL OF NUTRITION

GEORGE R. COWGILL, *Editor*

Yale University School of Medicine, New Haven, Conn.

HAROLD H. MITCHELL, *Associate Editor*

University of Illinois, Urbana, Ill.

## EDITORIAL BOARD

ALBERT G. HOGAN

ICIE MACY-HOOBLER

HOWARD B. LEWIS

ERNEST B. FORBES

HENRY A. MATTILL

ALFRED T. SHOHL

PHILIP A. SHAFFER

LELA E. BOONER

GEORGE O. BURR

CONRAD A. ELVEHJEM

RUSSELL M. WILDER

VOLUME 22

JULY — DECEMBER, 1941

Linlithgow Library.  
Imperial Agricultural Research Institute.  
New Delhi.

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY  
PHILADELPHIA, PA.



# C O N T E N T S

No. 1 JULY, 1941

DAVID M. GREENBERG AND WILBUR DOANE MILLER. Severe calcium deficiency in growing rats. III. Serum calcium of individual animals during development of calcium deficiency. One figure . . . . .	1
HAROLD R. STREET, GEORGE R. COWGILL AND H. M. ZIMMERMAN. Further observations of riboflavin deficiency in the dog. Two figures . . . . .	7
M. BODANSKY AND VIRGINIA B. DUFF. Dependence of fetal growth and storage of calcium and phosphorus on the parathyroid function and diet of pregnant rats . . . . .	25
H. S. WILGUS, JR., F. X. GASSNER, A. R. PATTON AND R. G. GUSTAVSON. The goitrogenicity of soybeans . . . . .	43
CARROLL F. SHUKERS, ELIZABETH M. KNOTT AND FREDERIC W. SCHLUZT. Magnesium balance studies with infants. Two figures . . . . .	53
RALPH HOAGLAND AND GEORGE G. SNIDER. Nutritive properties of steam rendered lard and hydrogenated cottonseed oil . . . . .	65
ROY LAVER SWANK AND OTTO A. BESSEY. III. Avian thiamine deficiency Characteristic symptoms and their pathogenesis. One figure . . . . .	77
MARGARET LAWRENZ AND H. H. MITCHELL. The effect of dietary calcium and phosphorus on the assimilation of dietary fluorine . . . . .	91

No. 2 AUGUST, 1941

H. D. ANDERSON AND A. L. MOXON. The excretion of selenium by rats on a seleniferous wheat ration. Two figures . . . . .	103
R. W. ENGEL AND W. D. SALMON. Improved diets for nutritional and pathologic studies of choline deficiency in young rats. Two plates (five figures) . . . . .	109
THEODORE F. ZUCKER, LILIAN HALL, MARGARET YOUNG AND LOIS ZUCKER. The growth curve of the albino rat in relation to diet. Four figures . . . . .	123
THEODORE F. ZUCKER, LILIAN HALL AND MARGARET YOUNG. Growth and calcification on a diet deficient in phosphate but otherwise adequate. Four figures . . . . .	139

W C SHERMAN. The effect of certain fats and unsaturated fatty acids upon the utilization of carotene Two figures . . . . .	153
LORIN E. HARRIS AND H. H. MITCHELL The value of urea in the synthesis of protein in the paunch of the ruminant. I In maintenance Two figures	167
LORIN E. HARRIS AND H H MITCHELL. The value of urea in the synthesis of protein in the paunch of the ruminant. II. In growth . . . . .	183
HELEN G. OLDFHAM The effect of heat on the availability of the iron of beef muscle Two figures . . . . .	197
M. MASON GUEST. Carbohydrate storage and mobilization in the rat. Five figures . . . . .	205

## No. 3 SEPTEMBER, 1941

RICHARD H FOLLIS, JR., HARRY G DAY AND E. V. MCCOLLUM. Histological studies of the tissues of rats fed a diet extremely low in zinc Two plates (eleven figures) . . . . .	223
WENDELL H. GRIFFITH. <i>Editorial Review.</i> The nutritional importance of choline . . . . .	239
SIDNEY B. FINN AND HAROLD C HODGE Reduction in experimental rat caries by fluorine. Six figures . . . . .	255
ARTHUR D. HOLMES, FRANCIS TRIPP, E. A. WOELFFER AND G HOWARD SATTERFIELD The ascorbic acid content of cow's milk during pregnancy	267
H G BAROTT AND EMMA M PRINGLE Energy and gaseous metabolism of the hen as affected by temperature Seven figures . . . . .	273
R A RUSSELL AND E. S. NASSET. The effects of various vitamin supplements and of whole yeast on the digestion and absorption of the carbohydrate of a complete diet. One figure . . . . .	287
BARNETT SURE. Further observations on riboflavin as a food factor in economy of food utilization. One figure . . . . .	295
HARRY J. DEUEL, JR., NELLIE HALLIDAY, LOIS F HALLMAN, CORNELIA JOHNSTON AND ALBERT J MILLER. The production of high vitamin A milk by diet . . . . .	303
THOMAS H. JUKES The effect of certain organic compounds and other dietary supplements on perosis Two figures . . . . .	315
HSUEH-CHUNG KAO, R T. CONNER AND H. C SHERMAN Influence of protein intake upon growth, reproduction, and longevity studied at different calcium levels . . . . .	327

## CONTENTS

v

## No. 4 OCTOBER, 1941

RAY TREICHLER AND H. H. MITCHELL	The influence of plane of nutrition and of environmental temperature on the relationship between basal metabolism and endogenous nitrogen metabolism subsequently determined	333
JAMES H. SHAW AND PAUL H. PHILLIPS	The pathology of riboflavin deficiency in the rat Sixteen figures . . . . .	345
J. C. FORBES.	Vitamin B complex and fat metabolism . . . . .	359
ESTHER M. PATCH.	Dietary production of cataracts in larval <i>Amblystoma tigrinum</i> . One plate (nine figures) . . . . .	365
R. B. FRENCH, J. I. ROUTH AND H. A. MATTILL	The influence of previous regimes of protein feeding on the endogenous nitrogen metabolism of rats. One figure . . . . .	383
F. J. MCCLURE.	Observations on induced caries in rats. III. Effect of fluoride on rats' caries and on composition of rats' teeth. Two figures	391
HAROLD R. STREET.	Studies on the rat growth assay method for riboflavin. One figure . . . . .	399
W. J. DANN AND PHILIP HANDLER.	The nicotinic acid and coenzyme content of the tissues of normal and blacktongue dogs . . . . .	409
SAMUEL H. EPPSTEIN AND SERGIUS MORGULIS	The minimum requirement of rabbits for dl- $\alpha$ -tocopherol. Two figures . . . . .	415
F. L. BILLINGS, JACOB BIELY, HERBERT FISHER AND CARL HEDREEN.	The riboflavin content of fish products . . . . .	425
H. S. OLcott AND T. D. FONTAINE	The effect of autoclaving on the nutritive value of the proteins in cottonseed meal. Two figures . . . . .	431

## No. 5 NOVEMBER, 1941

JOHN R. FOY AND LEOPOLD R. CERECEO.	Differences in the behavior of rats and mice towards deficiencies of certain members of the vitamin B complex. Five figures . . . . .	439
MARGARET LAWRENZ AND H. H. MITCHELL	The assimilation of fluorine by rats from natural and synthetic cryolite and from cryolite-sprayed fruits	451
MARIANNE GOETTSCH AND ALWIN M. PAPPENHEIMER	$\alpha$ -Tocopherol requirement of the rat for reproduction in the female and prevention of muscular dystrophy in the young . . . . .	463
MARGARET L. FINCKE.	The utilization of the calcium of cauliflower and broccoli	477
ESMOND E. SNELL AND ERNESTINE QUARLES	The effect of incubation on the vitamin content of eggs . . . . .	483

## CONTENTS

BARNETT SURE	Dietary requirements for fertility and lactation. XXVIII. The lactation-promoting properties of cystine when added to casein diets	491
BARNETT SURE	Dietary requirements for fertility and lactation. XXIX The existence of a new dietary factor essential for lactation . . .	499
J. H. EBBS, F. F. TISDALL AND W. A. SCOTT.	The influence of prenatal diet on the mother and child . . . . .	515
AARON J. IHDE AND HENRY A. SCHUETTE.	Thiamine, nicotinic acid, riboflavin and pantothenic acid in rye and its milled products . . .	527
J. M. MCINTIRE, HARRY A. WAISMAN, LAVELL M. HENDERSON AND C. A. ELVEHJEM.	Nicotinic acid content of meat and meat products . . .	535

## No. 6 DECEMBER, 1941

H. H. MITCHELL AND T. S. HAMILTON, WITH THE TECHNICAL ASSISTANCE OF W. T. HAINES	The utilization by calves of the energy contained in balanced rations composed of combinations of different feeds	541
KLAUS UNNA, GRACE V. RICHARDS AND W. L. SAMPSON	Studies on nutritional achromotrichia in rats. Three figures . . .	553
CHARLES W. MUSHETT AND KLAUS UNNA	Inefficacy of hormones in nutritional achromotrichia of rats. One figure . . .	565
JOHN R. MURLIN, MARGARET E. MARSHALL AND CHARLES D. KOCHAKIAN	Digestibility and biological value of whole wheat breads as compared with white bread One figure . . . . .	573
ROBERT R. SEALOCK, DANIEL H. BASINSKI AND JOHN R. MURLIN.	Apparent digestibility of carbohydrates, fats, and "indigestible residue" in whole wheat and white breads. One figure . . . . .	589
M. E. YARBROUGH AND W. J. DANN.	Dark adaptometer and blood vitamin A measurements in a North Carolina nutrition survey. One figure . . .	597
C. F. HUFFMAN, C. L. CONLEY, C. C. LIGHTFOOT AND C. W. DUNCAN.	Magnesium studies in calves II. The effect of magnesium salts and various natural feeds upon the magnesium content of the blood plasma	609
MARGARET LAWRENZ AND H. H. MITCHELL	The relative assimilation of fluorine from fluorine-bearing minerals and food (tea), and from water and food . . . . .	621

# SEVERE CALCIUM DEFICIENCY IN GROWING RATS

## III. SERUM CALCIUM OF INDIVIDUAL ANIMALS DURING DEVELOPMENT OF CALCIUM DEFICIENCY<sup>1</sup>

DAVID M GREENBERG AND WILBUR DOANE MILLER

*Division of Biochemistry, University of California Medical School, Berkeley*

- ONE FIGURE

(Received for publication January 31, 1941)

The results of a study of the symptoms, the pathology, and the changes in chemical composition of rats suffering from uncomplicated, severe, calcium deficiency have recently been published by Boelter and Greenberg ('41). It was demonstrated, in the course of this study, that a marked reduction in the serum calcium concentration occurred. To demonstrate this fact it was necessary to sacrifice groups of animals at varying intervals of time, and analyse their pooled blood.

It seems highly desirable to follow the changes in serum calcium in the same animal during the whole course of the calcium deficiency. The successful application by Lindner and Kirk ('37) of the cerate-Mohr's salt titration procedure to the quantitative estimation of calcium in a single drop of blood serum made such an undertaking feasible, and led us to undertake the investigation.

The results obtained show that although there are considerable daily fluctuations, development of a severe calcium deficiency leads to a progressive decline in the serum calcium concentration. The serum protein concentration remains unchanged. It is interesting that even though the serum calcium level was greatly lowered, at no time did the rats on the low calcium regimen show any indications of tetany.

<sup>1</sup> Aided by a grant from the Christine Breon Fund for Medical Research. Technical assistance was furnished by the personnel of W P. A., official project no 65-1-08-62

## EXPERIMENTAL

The investigation was carried out upon eight rats of the Long-Evans strain reared in our stock colony. After being weaned at 28 days of age, the rats were kept on the stock colony diet for a week or more until they weighed about 100 gm. each. They were then placed on the calcium-low diet. This procedure was followed in order to have heavier animals that would stand the successive bleeding better than those that are ordinarily used in the calcium-deficient studies. The starting range in weight was from 94 to 125 gm., and the mean weight was  $107 \pm 12$  gm.<sup>2</sup> When they were sacrificed after 82 to 134 days on the low-calcium regimen, the mean weight was  $161 \pm 40$  gm. At this time all of the animals had dropped below their maximum weight which averaged  $199 \pm 40$  gm. The maximum weight was attained at anywhere from 32 to 68 days after the animals were placed on the deficient diet.

The experimental diet contained about 10 mg. of calcium per 100 gm. of food. Its composition has been reported in a previous publication (Kleiber, Boelter and Greenberg, '40).

The apparatus and technique of Kirk ('33) were used for the drop analysis. Blood, obtained at intervals of 2 to 6 days by snipping the tail, was collected in micro-centrifuge tubes and, after centrifugation, measured samples (about 0.05 ml.) of the separated serum were pipetted into 3 ml. capacity silica ware crucibles, and the pipettes, calibrated for capacity, were rinsed out with an equal volume of redistilled water. The serum was dried in an electric oven maintained at about 80°C. as a preliminary to ashing. The crucibles were then transferred to a micro-muffle furnace and the serum ashed by adjusting the temperature to 400-500°C. After 45 minutes of heating, the temperature was raised to 500-550°C. for about 10 minutes. The ash was dissolved in about 1 drop of 0.1 N hydrochloric acid, and the calcium precipitated as the oxalate in the silica crucible. The precipitate was washed

<sup>2</sup> The measure of variability used in this paper is  $\pm$  standard deviation.

by means of a sintered glass, micro external filter. After washing, the calcium oxalate was dissolved in a drop of 6 N sulfuric acid, and the oxalic acid oxidized by addition of a measured excess of standard ceric sulfate. The remaining cerate ion was titrated with ferrous ammonium sulfate from a capillary burette in the presence of o-phenanthroline as indicator. The analysis was carried out by following the procedure of Lindner and Kirk ('37) except for a few modifications which are given below.

With the exception of the ceric sulfate standard solution, which remained stable indefinitely, it was found desirable to prepare the solutions for use from carefully protected stock solutions and recrystallized salts about twice a month. The asbestos suspension for filtering the calcium oxalate was left to soak in the acid-ceric sulfate solution, and only a small quantity was prepared for immediate use as described by Lindner and Kirk. The o-phenanthroline indicator solution was added directly to the solution of the Mohr's salt (about 15 drops to 30 ml.) before the titration rather than being introduced into the solution containing the sample following the addition of the ceric sulfate. This procedure eliminates a blank for the titer of the indicator, since the o-phenanthroline is kept in the reduced stable form by the Mohr's salt.

To prevent gross errors, the analysis on such minute samples must be performed with great care to avoid contamination. In the present work all analyses were performed in duplicate, and the average difference between two duplicate analyses was about 4%. Occasional results were obviously in great error and these were discarded.

#### RESULTS

The curve of the change in blood serum calcium concentration with time on the low calcium diet is shown in figure 1. The analytical values for each experimental animal are indicated by a distinctive symbol. The solid line represents the average curve for all eight rats. It is to be observed that the calcium level commences to drop almost at once after

the animals are placed on the experimental regimen, although the concentration does not fall below the normal range until after about 2 weeks on the diet. After 30 days, the average serum calcium concentration has fallen to 7.4 mg. per 100 ml., and at 68 days it is 6.1. The rate of fall is greatest for the first 40 days, after which the serum calcium concentration tends to become stabilized. Rats killed at 82 and 133 days

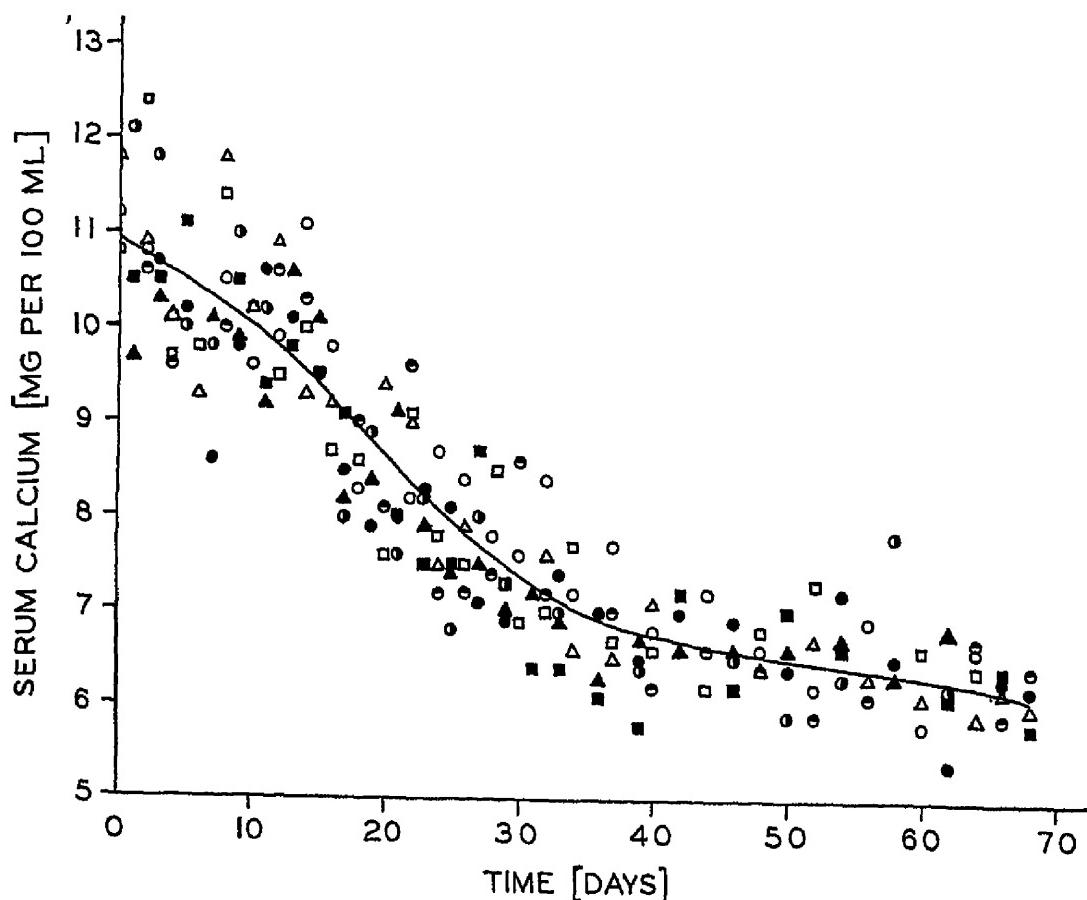


Fig 1 The change of the serum calcium concentration with time in individual rats on a diet very low in calcium.

showed essentially the same serum calcium concentration as is given by the curve at 68 days.

The serum protein level is not changed by the calcium deprivation. Analysis of the serum protein on a series of nine calcium deficient rats by the method of Greenberg and Mirolubova ('36) gave an average value of  $6.65 \pm 0.85$  gm. protein per 100 ml. of serum.

After 9 weeks on the diet, some of the animals were subjected to a galvanic shock, producing paralysis and symptoms of hemorrhage (Boelter and Greenberg, '41), but this caused no noticeable change in the serum calcium concentration. The animals given the galvanic shock lost a great deal of weight, which is one of the reasons the mean final weight is lower than the mean maximum weight of the animals.

Analyses of body calcium and phosphorus were carried out on five of the animals after they were sacrificed. This yielded values of  $0.426 \pm 0.06\%$  calcium and  $0.383 \pm 0.03\%$  phosphorus as against the normal values of 1.0 and 0.75%, respectively. The comparison shows that a great reduction in body calcium and phosphorus occurs on the low calcium diet. Depletion of the calcium stores of the animal apparently is necessary to produce a lowering of the serum calcium concentration in rats with an intact thyroid-parathyroid apparatus.

#### SUMMARY

1. The change in serum calcium concentration in rats on a severely calcium deficient diet has been followed in the same animal during the whole course of the deficiency by means of analysis upon a single drop of blood serum.
2. Development of severe calcium deficiency resulted in a progressive decline in the serum calcium until the concentration of calcium fell to about 6 mg. per 100 ml. of blood serum. The serum protein concentration was not altered by calcium deprivation.

We are greatly indebted to Dr. M. D. D. Boelter for carrying out the analysis of the serum protein and the body calcium and phosphorus.

#### LITERATURE CITED

- BOELTER, M D D, AND D M GREENBERG 1941 Severe calcium deficiency in growing rats I. Symptoms and pathology II Changes in chemical composition J Nutrition, vol 21, p. 61
- GREENBERG, D M, AND T N MIROLUBOVA 1936 Modifications in the colorimetric determination of the plasma proteins by the Folin phenol reagent J Lab and Clin Med, vol 21, p. 431.

- KIRK, P. L. 1933 Quantitative drop analysis I General apparatus and technique. *Mikrochemie*, vol 14, p 1
- KLEIBER, M., M D. D. BOELTER AND D M. GREENBERG 1940 Fasting catabolism and food utilization of calcium-deficient rats. *J Nutrition*, vol 19, p 517.
- LINDNER, R, AND P. L KIRK 1937 Quantitative drop analysis VII. Further investigations on the determination of calcium. *Mikrochemie*, vol. 22, p 291.

## FURTHER OBSERVATIONS OF RIBOFLAVIN DEFICIENCY IN THE DOG<sup>1</sup>

HAROLD R STREET,<sup>2</sup> GEORGE R COWGILL AND H. M ZIMMERMAN

*Departments of Physiological Chemistry and Pathology, Yale University School of Medicine, New Haven, Connecticut*

### TWO FIGURES

(Received for publication December 30, 1940)

Street and Cowgill ('39) reported certain observations of riboflavin deficiency in the dog, but numerous other phenomena remained to be described. These are presented in the present paper. Among other effects, degenerative changes in the nervous system have been observed.

### EXPERIMENTAL PROCEDURE

The animals used for this study were fifteen adult mongrel dogs, ranging in weight from 4.85 to 8.6 kg. at the beginning. All were given a vermifuge before the experiment began.

*Diet employed.* The basal diet used has already been published (Street and Cowgill, '39). Certain other pertinent details to be mentioned are as follows: The control animals received as a supplement to the basal diet 25 µg. of riboflavin<sup>3</sup> per kilogram of body weight daily. The provision of the water-soluble vitamins needed by the dog other than riboflavin was effected by the administration of a tikitiki prepared by ourselves. Growth tests with rats indicated that

<sup>1</sup> The expenses of this investigation were defrayed by grants from the Rockefeller Foundation and the Research Fund of the Yale University School of Medicine

<sup>2</sup> Vitamin Research Fellow, 1936-1938

<sup>3</sup> Obtained from the Borden Company, Bainbridge, New York, as crystalline lactoflavin, PX grade (highest purity).

this product contained only about 3 to 4  $\mu\text{g}$ . of riboflavin per cubic centimeter; at the level of 0.5 cc. per rat daily, this tikitiki, given as the sole source of water-soluble vitamins, permitted only poor growth, i.e., an average of 0.77 gm. per day during the first 8 weeks, for fifteen rats. Most of these animals developed moderate to extensive loss of hair, characteristic of flavin deficiency, as well as other symptoms of an insufficient supply of flavin. Six rats given daily 0.5 cc. of the tikitiki plus 40  $\mu\text{g}$ . of crystalline riboflavin as their source of the water-soluble vitamins grew at the reasonably satisfactory rate of 2.66 gm. per day during a period of 8 weeks. These animals appeared completely normal in every respect.

The tikitiki, when assayed for vitamin B<sub>1</sub> by the pigeon method (Block, Cowgill, and Klotz, '32) was found to contain 3.3 pigeon units per cubic centimeters, or approximately 8 international units per cubic centimeter. It was fed to the dogs in the amount of 0.5 cc. per kilogram of body weight daily (4 I.U./kg.) which is about three times the amount of vitamin B<sub>1</sub> estimated to be required by dogs of this size (Cowgill, '34).

*Grouping of animals.* The dogs used in this study may be classified into three groups, according to the dietary regimens upon which they subsisted.

Group 1. Five dogs were given only the basal diet until the development of sudden collapse characteristic of dogs maintained on this ration (Street and Cowgill, '39). Four of the animals, after collapse, were injected with a solution of riboflavin, the dose used being approximately 0.75 mg. per kilogram of body weight. Three of the dogs recovered and were then continued on the basal diet without further riboflavin until they collapsed for the second time. The second collapse was fatal in each case, although two of the dogs were injected with riboflavin late in the course of the attack.

Group 2. Four dogs were given small daily doses of flavin (4 to 8  $\mu\text{g}$ . per kilogram of body weight), amounts which were thought to be sufficient to support life but not enough to main-

tain health. Thus, it was hoped, a condition of chronic deficiency would be produced. The dogs of groups 1 and 2 were offered daily an amount of the basal mixture furnishing approximately 90 calories per kilogram of body weight.

Group 3. Six dogs served as inanition controls for certain of the dogs of the two preceding groups. Each control was paired with an experimental animal of similar body weight and was given daily that amount of the basal mixture eaten by the experimental partner the preceding day. In addition, the control dogs received daily 25 µg. of riboflavin per kilogram of body weight. Thus, the only difference in the treatment of the experimental and control dogs was the amount of riboflavin given as a supplement to the basal diet.

*Clinical observations.* The animals were weighed weekly and were examined frequently for any abnormalities such as loss of hair, sore mouth, dermatitis, cataract, or corneal opacity. Each dog was taught to run on a turn table, perform on an obstacle board, and stand on its hind legs; records were kept throughout the experiment of any changes in the ability to perform these tasks. The dogs that developed acute riboflavin deficiency were autopsied immediately after death. The animals of group 2, as well as the control dogs, were chloroformed and autopsied at the conclusion of the experiment.

*Histologic technique.* The brain and spinal cord and the sciatic, brachial, and usually the vagus nerves were fixed immediately after removal in 95% alcohol, 10% neutral formalin and Müller's solution (without formaldehyde). The nervous tissues were stained by the Marchi, Sudan III, Kultschitzky and Nissl techniques. Sections of the liver and of some of the other viscera were stained with hematoxylin and eosin.

#### RESULTS

*General behavior of the animals.* We have previously described ('39) the behavior of dogs maintained on the basal diet alone. All such animals collapsed in a characteristic manner, usually in from 100 to 150 days after beginning the

experiment. Animals will recover from this state of collapse only if given riboflavin before the attack has progressed too far. Aside from the collapse, the chief effects of the diet observed in the animals of group 1 were lessened activity and a moderate decrease in appetite. The dogs of group 2 also exhibited decreased appetite and activity after a considerable period.

In contrast to the symptoms seen in rats on such a diet, loss of hair was not observed in these animals except that the hair wore thin on the chests of two dogs after they became inactive and spent most of the time lying down. No soreness of the mouth was seen except in two dogs. Experimental dog 7 and control dog 8 both developed a unilateral necrotic bleeding area on the mucosa of the cheek opposite the last upper molars. It was the opinion of Dr. Bert G. Anderson<sup>4</sup> that the ulcers were an effect of the irritation of the cheek by the thick deposits of calculus on these teeth. Removal of the calculus from the teeth of one of the dogs caused temporary improvement.

The skin of the dogs in group 1 (acute deficiency) remained healthy. However, two of the animals in the chronic deficiency group (dogs 9 and 13) exhibited a persistent scaling of the skin of the abdomen and medial surface of the hind legs, similar to that described by Sebrell and Onstott ('38). Large, thin, loose scales of skin were constantly present. This was accompanied by a very slight erythema.

With a few exceptions, the animals lost considerable weight; these weight changes are shown in table 1. It will be seen that in the acute deficiency group the weight losses were

TABLE 1  
*Weight losses in riboflavin deficiency*

DOG NO.	GROUP 1 ACUTE DEFICIENCY					GROUP 2 CHRONIC DEFICIENCY					GROUP 3 CONTROL				
	1	8	53	55	57	7	9	13	51	2	6	8	10	12	52
Initial weight (kg.)	8.6	7.3	6.0	7.9	7.4	7.2	6.0	4.9	6.0	8.6	7.5	7.1	6.6	5.8	6.6
Final weight (kg.)	6.0	9.0	3.6	5.3	4.8	5.2	5.9	3.7	4.9	7.3	5.4	4.1	7.2	5.0	5.0
Weight loss (%)	30	..	40	33	41	28	2	25	18	15	28	42	..	14	24

\* Associate Professor of Surgery, Yale University School of Medicine.

from 30 to 41%, except for one animal which gained weight. In the chronic deficiency group three of the dogs lost 19 to 28%. The weight of dog 9 for the major part of the experiment remained close to the initial weight of 6.0 kg. There was, however, a period during which the weight dropped slowly to 4.75 kg. The weight at the end of the experiment was 5.85 kg. The six control dogs lost from 13 to 42% of their weight, with the exception that dog 10 gained 0.6 kg. This gain in weight on a limited food intake was in part explained by the fact that this particular animal was very inactive.

*Condition of the eyes.* Five dogs showed an abnormal condition of the eyes; three of these were in group 1 (acute deficiency), two in group 2 (chronic deficiency). No abnormalities were seen in the eyes of the control dogs.

Group 1. On the one hundred and thirty-sixth day of the experiment it was observed that the eyes of dog 3 were half-closed, the nictitating membranes pulled forward until they covered about a third of the eye, and the eye balls rotated dorsally. On the one hundred and thirty-eighth day the eyes were no longer rotated this way, but the eyelids were still half-closed. On the one hundred and fortieth day the condition was the same. On the one hundred and fiftieth day and for the remainder of the experiment the eyes looked normal. It is to be noted that the dog appeared on the verge of collapse on the one hundred and thirty-sixth day, remained inactive for 4 days, then spontaneously recovered much of its vigor by the one hundred and forty-fifth day.

In the case of dog 55, it was observed that the left eye was half-closed on the one hundred and fifth day. This condition disappeared after 2 days. On the one hundred and twenty-first day it was noticed that the right eye was kept partially closed. This also returned to normal within a few days. On the one hundred and eightieth day it was found that there were opacities of the cornea in both eyes, that in the left eye measuring about  $3 \times 4$  mm., the measurements of that in the right eye being somewhat smaller.

In dog 57 on the two hundred and sixth day of the experiment, the day preceding death, it was observed that the right cornea had a dry appearance and was indented in many places, giving a pebbled effect. There was no perceptible opacity and there did not appear to be any breakdown of the surface. The left eye appeared perfectly normal.

Group 2. Opacities of both corneas were observed in dog 7 on the three hundred and ninety-seventh day, measuring about 3 mm. by 1 mm. in the left eye and a little smaller in the right. By the four hundred and eleventh day the opaque spot in the right cornea had increased somewhat in size. The condition was described by Dr. Edward N. DeWitt<sup>5</sup> on the four hundred and fifty-fourth day as follows: "There are opacities in the stroma layer of the cornea of each eye, and scattered for 2 or 3 mm. beyond the main mass are small dot-like opacities in each eye which remind one of deep punctate keratitis. The rest of each eye is perfectly normal." In the left eye the opacity measured 5 mm. horizontally by 2.5 mm. vertically, and in the right eye 4 mm. horizontally by 2 mm. vertically. It was found on the four hundred and ninety-first day that the left eye was watering, and by the five hundred and thirty-ninth day both eyes were watering, forming brown stains on the animal's white coat running down from the anterior corner of each eye.

Opacities were discovered in the eyes of dog 13 on the two hundred and sixty-fifth day of the experiment. They appeared as sharply circumscribed light spots in the center of each cornea, measuring about 3 to 4 mm. in the transverse direction by about 1.5 mm. vertically. On the two hundred and ninety-third day there was an area of opacity around the original spot in the left eye: the outer area was not so intensely opaque, but paler. Both eyes were watering considerably.

Doctor De Witt described the appearance of the eyes on the three hundred and sixty-fourth day in the following words:

<sup>5</sup> Assistant Clinical Professor of Ophthalmology, Yale University School of Medicine.

"Left eye: Externally the eye is normal. There is a normal reaction to light. There seems to be a slight epiphoria. *Cornea*: external epithelial layer normal and clear. In the stroma tissue there is an opacity measuring horizontally 5 mm., vertically 2.5 mm. Its general shape is oval, and it has a glistening, shining, water-like sheen. Toward the inner canthus the opacity is somewhat denser, and has a yellowish glistening hue, which resembles early calcareous degeneration. *Anterior chamber*: normal in depth and clear. *Lens*: normal, perfectly clear, no opacities. *Vitreous, choroid, retina, nerve-head, vessels* normal. *Right eye*: The external appendages are the same as those of the left. There is an opacity in the stromal tissue of the cornea resembling the one in the left eye, but it is much smaller, measuring 3 mm. horizontally and 2 mm. vertically."

From the four hundred and first day till the end of the experiment on the four hundred and fifty-seventh day the eyes were watering noticeably, and on one occasion were found to be stuck shut in the morning.

*The effect of varying the amount of riboflavin given to the animals of group 2.* Dog 7 received daily 55 µg. (8 µg./kg.) from the thirty-fifth to four hundred and thirty-fourth day, and 28 µg. (4 µg./kg.) from the four hundred and thirty-fifth to five hundred and fortieth day. Dog 9 received daily 48 µg (8 µg./kg.) from the twenty-fourth to two hundred and sixty-seventh day, 25 µg. (4 µg./kg.) from the two hundred sixtieth-eighth to three hundred and twenty-third day, 48 µg. (8 µg./kg.) from the three hundred and twenty-fourth to four hundred seventy-third day, and 35 µg. (6 µg./kg.) from the four hundred and seventy-fourth to four hundred and ninety-third day.

In addition, the following single injections were given to dog 9: 2.25 mg. on the three hundred and thirty-second day, 1 mg. on the three hundred and seventy-first day, 1 mg. on the four hundred and second day.

Dog 13 was fed the basal diet only from the first to one hundred and forty-ninth day; then it received 20 µg. (4 µg./kg.) of riboflavin from the one hundred and fiftieth to three hundred and sixty-first day; and the basal diet only from the three hundred and sixty-second to four hundred and fifty-seventh day.

Dog 51 was given 48 µg. (8 µg./kg.) of riboflavin from the eighth to one hundred and ninety-second day, and 25 µg. (4 µg./kg.) from the one hundred and ninety-third to two hundred and eighth day.

Since dog 9, after 267 days, had maintained its weight at the initial level of 6.0 kg. while receiving 8 µg. of riboflavin per kilogram, it was decided to cut the flavin dosage in half. The animal at once began to lose weight (fig. 1) and soon became less active. The dose of flavin was increased to 8 µg./kg.

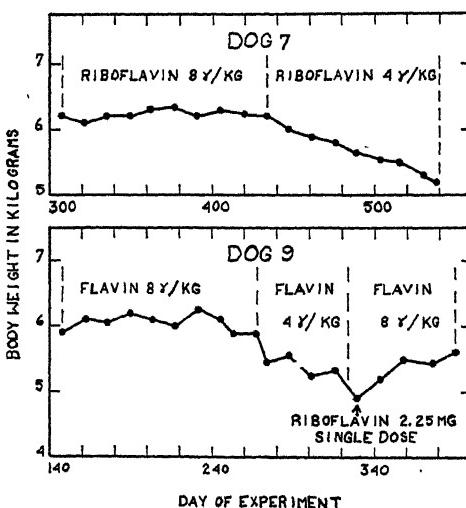


Fig. 1 Graph showing the loss in weight of two dogs when the daily dose of riboflavin was reduced to 4 micrograms per kilogram of body weight after a long period of maintenance with 8 micrograms of riboflavin per kilogram.

again on the three hundred and twenty-fourth day, but despite this, the dog developed the characteristic collapse of acute riboflavin deficiency on the three hundred and thirty-second day. It was restored by the injection of 2.25 mg. of riboflavin.

The riboflavin supplementation for dog 7 was reduced from 8 µg./kg. to 4 µg./kg. on the four hundred and thirty-fifth day, to see whether it would be possible to hasten the development of certain symptoms of insufficient flavin which had appeared

in an early form. Up to this time the weight had been constant at 6.1 to 6.3 kg. for the preceding 125 days. The body weight at once began to decline, gradually but steadily, decreasing to 5.2 kg. by the five hundred and fortieth day. These weight changes are shown graphically in figure 1.

Dog 13 behaved in a somewhat anomalous manner; although given no flavin for the first 149 days the characteristic collapse did not appear; the animal did lose a little weight. Supplementation with 4 µg. riboflavin per kilogram for the next 212 days prevented any appreciable further loss of weight. This dog was occasionally observed to eat its feces. Presumably this explains her unusually low requirement for riboflavin, since it has been our experience that coprophagy protects dogs from deficiency of the water-soluble vitamins.

#### *Neurological dysfunctions*

Numerous observations were made of the ability of the animals to perform on an obstacle board and a turn table as well as to balance themselves on their hind legs. The type of information yielded by these tests and the interpretation placed on them by a clinical neurologist are illustrated in the remarks of Dr. James C. Fox, Jr.,<sup>6</sup> concerning animals 7, 9 and 13 in the chronic deficiency group and control dog no. 8 presented in table 2. In the acute deficiency dogs of group 1 some earlier signs of neurological dysfunction were noted in some but not all of the animals usually after about the ninetieth day of the dietary regime (dogs 3, 53 and 55). In contrast to these findings may be cited the fact that the group of control dogs remained in apparently perfect health throughout the experiment (see no. 8 in table 2); they were lively, had good appetites, and showed no neurological abnormalities although observations were made as late as the five hundred and thirty-fifth day of the experiment.

#### *Histologic studies*

*Nervous system.* A summary of the findings pertaining to the pathology of the nervous system is presented in table 3.

<sup>6</sup> Clinical Professor of Neurology, Yale University School of Medicine.

TABLE 2  
*Illustrative reports of neurological examination of animals*

DOG NO.	REMARKS
<i>Chronic riboflavin deficiency</i>	
7	4/7/38—426th day: "The dog gives the impression of being a little asthenic without true muscular weakness or ataxia in any of the tests. Treads and stands on hind legs well; reflexes active."
	6/25/38—505th day: "The dog has definitely changed since last examined on 4/7/38. The ordinary gait appears normal in contrast to that exhibited on the turn table which is abnormal. Throws hind legs on obstacle board; slides on turn table; refuses to stand on hind legs. Wavers when supported by fore paws and is a bit insecure in jumping. Fore paws apparently not affected. Shaking reaction is normal. Deep reflexes all diminished markedly, the knee jerks just barely obtained. <i>Impression:</i> Weakness in hind legs. The loss of the deep reflexes suggests lower neuron involvement. Whether there is any ataxia of higher origin cannot be determined; probably not."
	7/25/38—535th day: "Performance in general is similar to that described on 6/25/38. He does stand on his hind legs, insecurely, today. The deep reflexes cannot be obtained anywhere, even in the proximal portions of the limbs. When supported by the hind legs, the fore paws do not seem to have full strength. Although the unsteadiness was not increased significantly on the turn table, obstacle board, or when standing on the hind legs, he is definitely atactic on shaking. He does not object to pin prick or hair pulling anywhere."
9	4/7/38—411th day: "The dog is rather pepless, sluggish on obstacle board, keeps a rather broad base, is able to stand on its hind legs, treads slowly, and is not thrown off balance easily. Deep reflexes are normal."
	6/25/38—490th day: "This dog has changed quite markedly since last examined on 4/7/38. It is generally weak, sluggish, short of breath on exertion; soon sits down and then rests on the floor. Weakness in the hind legs increases the longer it is upright, the 'compass gait' becoming more striking. It is unable to hurdle, and treads very clumsily. Deep reflexes are practically absent except for a feeble jerk in the proximal flexors. Shaking: not particularly atactic but, in fact, quite stable in view of weakness of limbs. <i>Impression:</i> Lower motor neuron weakness, in both front and hind legs, and greater in the latter; generalized asthenia, and lowered cardiac reserve. It is very doubtful whether it has any ataxia of higher origin."
13	4/7/38—336th day: "This dog is in better condition neurologically than its two experimental colleagues, nos. 7 and 9. The impression is gained that it shows minimal lag and clumsiness of the hind legs on the obstacle board. This may be due to beginning weakness. Its behavior in other respects is normal, especially in the ability to stand on its hind legs unsupported. The knee jerks are still very lively. <i>Impression:</i> Beginning weakness of hind legs?"
	7/25/38—445th day: "Response the same as described in above note; possibly the right rear leg gives way a little more than the left after fatigue on the turn table or from walking on hind legs. Deep reflexes all lively."

*Control*—Companion of dog no. 7 paired-fed with basal diet but received in addition daily 25 $\mu$ g./kg. of riboflavin.

- 8 4/7/38—426th day: "Decidedly more alert and active than experimental partner; can tread faster and jumps when standing on hind legs. Is faster over obstacle board and slips a little less."
- 505th day: "No additional comments."
- 535th day: "Response is normal; reflexes active."

TABLE 3

*Myelin degeneration in the different groups of animals as revealed by various stains compared with the length of period of subsistence on the deficient diet*

DOG NO.	DAYS ON DIET	MARCHI		KULTSCHITZKY		SUDAN III		NISSL		PAIRED WITH EXPERIMENTAL DOG NO.
		Nerve	Cord	Nerve	Cord	Nerve	Cord	Cord	Brain	
Acute deficiency group										
53	110	—	—	—	—	—	—	—	—	
1	152	+	—	—	—	—	—	—	—	
55	182	3+	+	—	—	—	—	—	—	
57	207	4+	3+	3+	+	—	—	—	—	
Chronic deficiency group										
51	208	±	—	—	—	—	—	—	—	
13	457	3+	2+	2+	+	—	—	2+	—	
9	493	4+	4+	3+	3+	—	—	2+	—	
7	540	3+	3+	3+	3+	—	—	2+	—	
Control group										
2	158	+	—	—	—	—	—	—	—	1
6	131	—	—	—	—	—	—	—	—	
8	547	+	—	—	—	—	—	—	—	7
10	512	+	±	—	—	—	—	—	—	9
12	542	±	±	—	—	—	—	—	—	13
52	211	+	—	—	—	—	—	—	—	51

Grading system of lesions: — none; ± minimal or questionable; + mild or slight; 2+ moderate; 3+ extensive; 4+ very extensive or extreme.

As indicated in the earlier work on the pathologic changes in the nervous system in avitaminosis (Zimmerman and Burack, '34; Zimmerman, Cowgill and Fox, '37), the essential lesion is a demyelination of the peripheral nerves and the posterior columns of the spinal cord (fig. 2A and 2B). In the chronic deficiency state gliosis replaces the injured posterior columns. In each instance where cord changes are present the peripheral nerves are also affected, but the reverse is not always true. From this and the fact that the

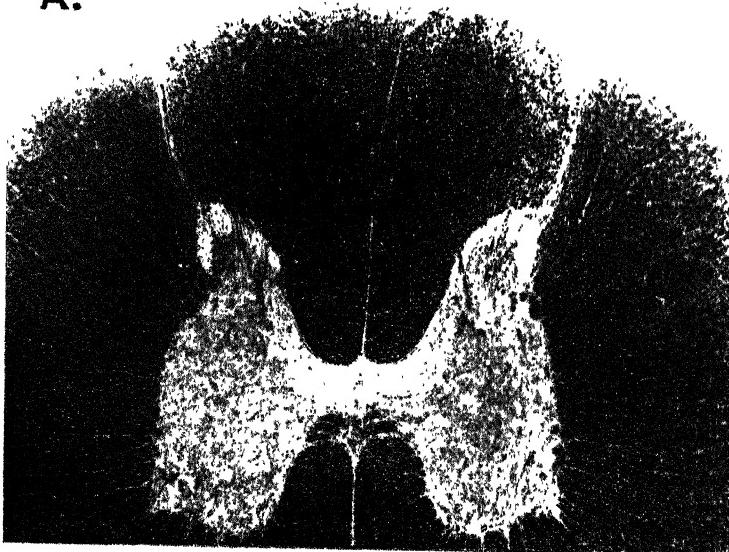
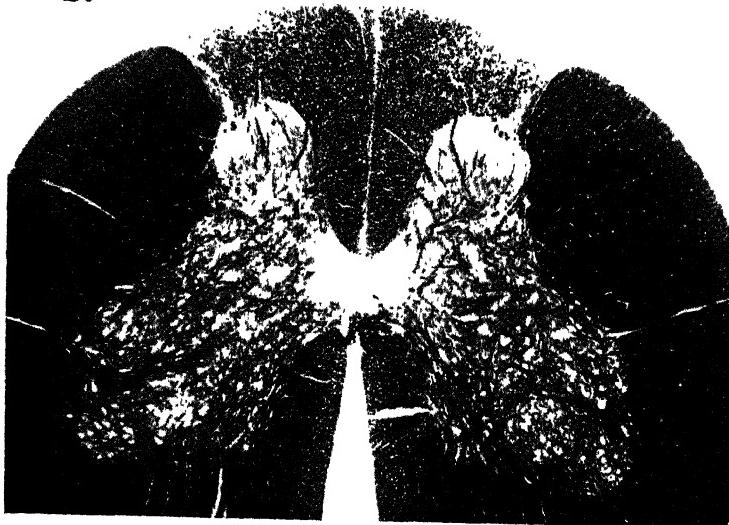
**A.****B.**

Fig. 2A Photomicrograph of transverse section of spinal cord of dog 9 showing very extensive (4+) destruction of myelin in posterior columns. Marchi stain.  $\times 7$ .

Fig. 2B Extensive (3+) demyelination of posterior columns in spinal cord of dog 7. Kultschitzky stain.  $\times 7$ .

nerve changes are usually more extensive than the cord lesions, it seems justifiable to assume that they precede the latter. It can also be shown quite clearly that the demyelinat-ing process extends centrally to the cord along the posterior nerve roots, the degree of demyelination becoming greater, the longer the subsistence on the deficient diet.

The presence of minimal or at most mild demyelination in the control animals suggests that the basal diet may have been slightly deficient in some other factor besides riboflavin. An alternative explanation is that our dosage of 25  $\mu$ g. of riboflavin daily was after all slightly inadequate. The first explanation is favored for the following reasons: In both experimental dog 1 and its control companion 2 the same mild degree of demyelination of peripheral nerves was ob-served after a period of only 152 and 158 days respectively, although the control animal was receiving 25  $\mu$ g. of riboflavin per kilogram daily as compared with only a trace for the experimental dog. It is difficult to believe that such widely different intakes of riboflavin could have had the same effect on the nerves. On the other hand, if a moderate deficiency of some other factor in the basal diet is postulated, both animals might be expected to show the same effect.

Another reason for this belief is that rats given tikitiki plus the liberal dosage of 40  $\mu$ g. riboflavin daily showed slight myelin degeneration. Some rats receiving tikitiki without riboflavin developed a more advanced yet still rather mild destruction of myelin. Because of the relatively mild form of the neuropathy in rats lacking riboflavin it seems highly improbable that rats receiving 40  $\mu$ g. of riboflavin daily, a level sufficient to prevent all outward manifestations of ribo-flavin deficiency, should develop nerve defects caused by lack of this factor.

*The liver.* In view of the report of Sebrell and Onstott ('38) that riboflavin deficiency in dogs is characterized by extremely fatty ("yellow") livers, it is of interest to report our findings. The livers of all animals in this study were examined macro-scopically at autopsy. Only three of these appeared abnormal.

The liver of dog 1 had a somewhat yellowish cast, while those of experimental dogs 9 and 53 were typical "yellow" fatty livers. Of five experimental dogs whose livers were examined histologically, three, dogs 13, 51, and 55 had normal livers, while those of dogs 7 and 53 were fatty but otherwise normal. Likewise, of three control animals examined, dogs 8, 10, and 52, all showed some increase in fat, but aside from this the microscopic appearance of the livers was normal. Thus, there was no consistent relationship of the condition of fatty livers to the riboflavin deficiency; both control and experimental animals exhibited this condition. It is pertinent to point out that our experiments were controlled by the use of inanition-control animals, whereas no controls are reported in the experiments of Sebrell and Onstott. It is indicated then, that the condition of fatty liver under discussion is related in some way to the partial inanition which cannot be entirely obviated when studying riboflavin deficiency, and not specifically to the lack of this factor. It is also possible, of course, that the differences in the two groups of observations are to be attributed to the differences in the basal rations employed.

*Hemoglobin and erythrocyte levels in the blood.* In our previous paper dealing with acute riboflavin deficiency it was reported that two out of three dogs developed lowered concentration of blood hemoglobin; the third animal gave normal values at all times. The anemia was characterized by little or no change in erythrocyte count, but a decrease in concentration of hemoglobin, the values for hemoglobin, which were 14 to 15 gm. per 100 cc. in the beginning, dropping to the level of 9 to 10 gm. per 100 cc. in one animal, and to 7 to 8 gm. in the other. We are now able to report concerning four additional dogs exhibiting the chronic picture of riboflavin deficiency and their respective inanition controls. In contrast to the observation of moderate anemia in the animals exhibiting the acute deficiency, it is a striking fact that the four animals, dogs 7, 9, 13, and 51, kept in a state of chronic deficiency for from 208 to 540 days, maintained concentrations of hemoglobin from 12 to 18 gm. per 100 cc. of blood, as

determined by bi-weekly examinations. While the values for hemoglobin tended to fluctuate a little, there was no upward or downward trend with the progress of the experiment. The hemoglobin values of the four inanition control animals behaved similarly. From this it would appear that when there is only a moderate but insufficient supply of riboflavin to the body, the hemopoietic system is spared, in contrast to other systems. Thus these dogs, after over 500 days of subsistence on the diet with insufficient supply of riboflavin, although exhibiting neuromuscular, ocular, and other manifestations of abnormality, nevertheless were maintaining erythrocyte counts and hemoglobin concentrations that were within accepted normal ranges.

*Gastric analyses.* Near the end of the experiment tests were made of the gastric contents of three chronically-deficient dogs (nos. 7, 9 and 13) for free and combined acid, and all values were within what are commonly regarded as normal limits. Evidently a long-standing moderate shortage of riboflavin cannot be regarded as having any specific effect on the gastric secretory mechanism.

#### DISCUSSION

In our previous paper dealing with acute riboflavin deficiency it was pointed out that marked deprivation with respect to this factor is associated with the development of a profound collapse in from about 100 to 150 days, which condition can be treated successfully by prompt administration of the missing factor. Such treated animals fail to show any specific neurologic disorders. The present paper, dealing with what might be called chronic riboflavin deficiency, makes it clear that long-continued subsistence on a diet containing some, but less than the minimal amount of, riboflavin required for perfect health, is not associated with collapse. Nevertheless, such a dietary regimen does result eventually in definite changes in the nervous system, appearing first as a demyelination of peripheral nerves which later progresses to involve the posterior columns of the spinal cord.

The fact that definite neurologically abnormal behavior developed in the present study so much later (after from 475 to 505 days) than in the earlier studies of Zimmerman and Burack ('34) and Zimmerman, Cowgill and Fox ('37) suggests that the diets used in these earlier studies were to some extent deficient in some other heat-stable factor of the B complex besides riboflavin. This would mean that the combination of vitamin B<sub>1</sub> concentrate prepared from rice polishings (Block and Cowgill, '32) and liver extract 343 powder <sup>7</sup> as used in the 1937 study, lacked some other water-soluble B-complex factor besides the riboflavin which was the intended variable of interest. Another reason that may be offered in support of this belief is the fact that in the 1937 study most of the dogs developed a secondary anemia which is in contrast to the observations made in the present investigation.

The fact that muscular weakness eventually appeared in the present chronically deficient animals suggests that the weakness which characterized the animals of the 1934 study, and which caused considerable difficulty at the time in detecting the presence of a true ataxia, thus leading to the perfection of technics such as use of the rotating table, obstacle board, training to walk on hind legs, etc., was indeed an expression of the marked shortage of riboflavin.

It may be pointed out that the neurologic symptoms of riboflavin deficiency in the dog are very different from those in vitamin B<sub>1</sub> deficiency. In the latter condition one of the first noticeable effects is the development of spasticity which causes a striking abnormality of gait. In contrast to this, dogs on a diet low in riboflavin continue to exhibit a normal or nearly normal gait, even when degeneration of the nervous system has progressed to the point where the deep reflexes are lost.

In pigs subsisting on faulty diets Hughes ('39) has reported the development of paralysis which could be cured by the administration of whey concentrate. Riboflavin was believed to be the effective agent in the concentrate, but it is not entirely clear that lack of vitamin B<sub>6</sub> was not playing a role. So far

<sup>7</sup> Lilly.

as it goes, Hughes' report may be regarded as confirmed by our present paper.

In view of the contributions by Day, Darby and Langston ('37) and Bessey and Wolbach ('38) on the appearance of corneal changes in rats deprived of riboflavin, the observations reported in the present paper constitute an extension of this to another species, namely, the dog.

Axelrod, Lipton and Elvehjem ('40) suggest that earlier work on riboflavin deficiency reported from this laboratory (Street and Cowgill, '39) probably represented other deficiencies as well because some of the animals developed a hypochromic anemia. While it is true that some of the animals with acute riboflavin deficiency developed a mild anemia, none of the animals in the chronic deficiency showed any tendency whatever toward anemia (for illustrations, see fig. 1). This may be explained in this way, that complete dietary lack of riboflavin results in some interference with hemoglobin formation, whereas with a small but incomplete amount of this factor the blood-forming tissues are able to perform fairly effectively. If the anemia appearing in a few of the animals were due to some complicating deficiency other than that of riboflavin, the anemia should have developed to a greater degree in the chronic study, since the experimental periods were so much longer.

#### SUMMARY

Prolonged subsistence on a diet low in riboflavin has led to the development of neurologic abnormalities in dogs, as evidenced by clumsiness in hurdling obstacles and in treading a revolving table, and finally by loss of the deep reflexes of the limbs. This was accompanied by myelin degeneration of peripheral nerves and the posterior columns of the spinal cord, becoming more extensive with the length of the period on the deficient diet. The administration of crystalline riboflavin to inanition control dogs maintained them in apparently perfect health, as judged during life, and largely prevented degenerative changes in the nervous system.

An increased fat content of the cells in otherwise normal livers was observed irregularly in the animals fed the inadequate diet; it was seen also in the inanition control dogs. This suggests that these changes in the liver are to be attributed to the accompanying inanition rather than specifically to the lack of riboflavin.

In the dog, as in the rat, the appearance of opacities of the cornea may be one of the effects of deprivation of riboflavin.

#### LITERATURE CITED

- AXELROD, A. E., M. A. LIPTON AND C. A. ELVEHJEM 1940 The production of uncomplicated riboflavin deficiency in the dog. *Am. J. Physiol.*, vol. 128, p. 703.
- BESSEY, O. A., AND S. B. WOLBACH 1938 Vascularization of the cornea of the rat in riboflavin deficiency. *Proc. Am. Assoc. Pathol. and Bacteriol.*, Thirty-eighth Ann. Meet.
- BLOCK, R. J., AND G. R. COWGILL 1932 The antineuritic vitamin: III. Removal of impurities by fractional precipitation. *J. Biol. Chem.*, vol. 97, p. 421.
- BLOCK, R. J., G. R. COWGILL AND B. H. KLOTZ 1932 The antineuritic vitamin. I. The method of assay, concentration of the vitamin with silver under various conditions, and its solubility in certain organic solvents. *J. Biol. Chem.*, vol. 94, pp. 765-782.
- COWGILL, G. R. 1934 The Vitamin B Requirement of Man. The Yale University Press, New Haven.
- DAY, P. L., W. J. DARBY AND W. C. LANGSTON 1937 The identity of flavin with the cataract-preventive factor. *J. Nutrition*, vol. 13, p. 389.
- HUGHES, E. H. 1939 The role of riboflavin and other factors of the vitamin-B complex in the nutrition of the pig. *J. Nutrition*, vol. 17, p. 527.
- SEBRELL, W. H., AND R. H. ONSTOTT 1938 Riboflavin deficiency in dogs. *U. S. Pub. Health Rep.*, vol. 53, p. 83.
- STREET, H. R., AND G. R. COWGILL 1939 Acute riboflavin deficiency in the dog. *Am. J. Physiol.*, vol. 125, p. 323.
- ZIMMERMAN, H. M., AND E. BURACK 1934 Studies on the nervous system in deficiency diseases. II. Lesions produced in the dog by diets lacking the water-soluble, heat-stable vitamin B<sub>2</sub>(G). *J. Exp. Med.*, vol. 59, p. 21.
- ZIMMERMAN, H. M., G. R. COWGILL AND J. C. FOX, JR. 1937 Neurologic manifestations in vitamin G(B<sub>2</sub>) deficiency. An experimental study in dogs. *Arch. Neurol. and Psychiat.*, vol. 37, p. 286.

# DEPENDENCE OF FETAL GROWTH AND STORAGE OF CALCIUM AND PHOSPHORUS ON THE PARA- THYROID FUNCTION AND DIET OF PREGNANT RATS<sup>1</sup>

M. BODANSKY AND VIRGINIA B. DUFF

*The John Sealy Memorial Research Laboratory and the  
University of Texas Medical School, Galveston*

(Received for publication February 21, 1941)

The work on which this report is based was planned with the object of determining the extent to which the composition of the rat fetus depends on the parathyroid function and intake of calcium and phosphorus of the maternal organism during pregnancy. To obtain accurate data of birth weights and composition, the newborn rats were either taken from the cages before there was an opportunity to nurse, or were removed from the uterus when there was unmistakable evidence that parturition had begun. The rat pups were wiped dry, weighed individually and a representative number was prepared for duplicate analyses. For each analysis two or more pups, weighing a total of approximately 10–12 gm., were each cut into several pieces, dried at 105°C. to constant weight in silica crucibles, then ashed in a muffle furnace. Calcium was determined by combining the method of McCrudden ('09-'10) with the titration procedure of Shohl and Pedley ('22). Phosphorus was determined by the method of Shohl and Brown ('32).

<sup>1</sup>This work was aided by generous grants from the Mead Johnson Company, Evansville, Indiana, and approved by the Council for Pediatric Research of the American Academy of Pediatrics.

## RESULTS

The largest number of observations was made on diet no. 7 (Ca 0.49%, P 0.49%) of Cox and Imboden ('36 a). Forty litters from parathyroidectomized rats were compared with thirty litters from unoperated control animals. The differences between these two groups are shown in table 1.

Comparison of total fetal storage of calcium and phosphorus emphasizes the difference between fetal development in normal and parathyroidectomized animals. The pups from the parathyropenic rats contained 30% less calcium than the control pups, while the fetal storage of calcium per litter was on an average less than half of that in the controls.

Rats fed diet no. 27 (Ca 0.122%, P 0.245%) of Cox and Imboden ('36 a) consumed during pregnancy approximately one-fourth of the calcium and one-half of the phosphorus ingested by the rats on diet no. 7. In spite of these differences, the offspring from both the control and parathyroidectomized rats on diet no. 27 were on an average almost identical in composition with those from the corresponding group fed diet no. 7. However, owing to the numerically larger litters produced by the controls on diet no. 27 than on diet no. 7, a result which is tentatively assumed to have been adventitious, the average fetal storage of calcium and phosphorus was actually higher in the former group than in the latter. On the other hand, in the parathyroid-deficient rats on diet no. 27 the litters were smaller than in the corresponding group on diet no. 7, a finding which may or may not have statistical significance, but which accounts, in part, for the difference in total fetal storage of calcium and phosphorus.

With diet no. 26 (Ca 0.017%, P 0.245%) it is practically impossible to breed parathyroidectomized rats successfully (Bodansky and Duff, '41 a). However, if before being placed on this diet such rats are fed diet no. 7 for a few days during the mating period, the proportion of pregnancies to matings is increased from about 3% to nearly 25%. In table 1 are summarized the data for ten litters from parathyroidectomized rats subjected to this routine, and, for comparison, ten

TABLE I

Influence of parathyroid deficiency and mineral composition of the diet on fetal development and fetal storage of calcium and phosphorus

Diet number	7	27	26	16	19	8	10	12
Condition of pregnant animals	N-C <sup>a</sup>	P-D <sup>a</sup>	N-C	N-C <sup>a</sup>	P-D <sup>a</sup>	N-C	P-D	N-C
Number of litters	30	40	13	12	14	10	13	5
Average number per litter	9.3	8.6	11.5	6.4	9.5	10.0	7.6	9.2
Standard deviation	±3.1	±2.3	±2.3	±3.1	±1.5	±1.4	±2.0	±0.7
Average litter weights, in gm.	49.62	29.19	56.15	21.01	45.12	52.40	33.24	41.60
Standard deviation	±15.38	±8.20	±11.20	±6.45	±16.96	±8.02	±9.85	±9.14
Average birth weight, in gm.	5.45	3.49	4.95	3.67	5.14	5.24	3.96	5.24
Standard deviation	±0.67	±0.83	±1.11	±0.98	±0.98	±0.93	±0.49	±0.72
Total solids, average %	13.51	12.93	13.49	12.88	13.41	13.17	12.75	12.92
Standard deviation	±0.54	±0.60	±0.83	±0.79	±0.32	±0.89	±0.52	±0.45
Ash, %								
Minimum	1.502	1.241	1.548	1.284	1.518	1.574	1.207	1.304
Maximum	1.962	1.474	1.838	1.734	1.852	1.732	1.590	1.650
Mean	1.675	1.474	1.678	1.470	1.701	1.363	1.411	1.503
Standard deviation	±0.108	±0.211	±0.106	±0.144	±0.103	±0.046	±0.142	±0.095
Calcium, %								
Minimum	0.193	0.094	0.189	0.126	0.157	0.210	0.069	0.102
Maximum	0.301	0.269	0.318	0.285	0.275	0.295	0.225	0.231
Mean	0.239	0.166	0.240	0.109	0.220	0.236	0.151	0.172
Standard deviation	±0.028	±0.049	±0.035	±0.049	±0.034	±0.018	±0.048	±0.038
% of ash, mean	14.22	11.21	14.24	11.37	13.62	14.20	10.55	11.43
Standard deviation	±1.05	±1.98	±1.38	±2.21	±1.36	±1.06	±0.43	±1.92
Phosphorus, %								
Minimum	0.259	0.210	0.232	0.213	0.258	0.292	0.224	0.233
Maximum	0.336	0.338	0.343	0.318	0.346	0.308	0.301	0.325
Mean	0.295	0.271	0.291	0.273	0.296	0.320	0.263	0.281
Standard deviation	±0.012	±0.037	±0.039	±0.039	±0.023	±0.008	±0.025	±0.024
% of ash, mean	17.64	18.34	17.31	18.54	17.41	17.58	18.64	16.89
Standard deviation	±0.99	±1.19	±1.19	±1.45	±0.91	±0.49	±0.72	±1.00
Calcium : Phosphorus ratio in ash <sup>b</sup>	0.81	0.61	0.82	0.62	0.78	0.81	0.57	0.68
Fetal storage,								
total, per litter;								
Calcium, mean, in mg	117.4	50.1	136.7	35.0	106.7	124.6	35.0	71.5
Standard deviation	±37.5	±22.6	±35.6	±12.5	±43.1	±26.9	±20.2	±16.8
Phosphorus, mean,	145.5	79.4	164.4	57.0	134.8	153.5	61.1	106.6
Standard deviation	±44.3	±92.1	±43.2	±17.7	±51.1	±27.0	±27.0	±19.2

<sup>a</sup> Based on mean values for per cent calcium and phosphorus.

<sup>b</sup> P-D denotes parathyroidectomized rats. <sup>c</sup> Based on mean values for per cent calcium and phosphorus.

Owing to the extremely low rate of reproduction in the parathyroid-deficient rats, the procedure was adopted of mating these animals and a corresponding number of unoperated controls on diet no. 26 on which they remained for the greater part (usually 14-20 days) of the gestation period.

litters from unoperated rats submitted, similarly, to a preliminary period (7 days) of feeding on diet no. 7. In addition, data are included for fourteen litters from unoperated rats which, during mating and pregnancy, were fed diet no. 26 exclusively.

Examination of the results for the control animals on diets nos. 26, 27, and 7, reveals that even severe calcium deprivation produced comparatively little change in the birth weight or composition (total solids, ash, calcium, and phosphorus) of the young. The total fetal storage of calcium and phosphorus fell within nearly the same range of variation on calcium-deficient diet no. 26, as on diet no. 7, which was presumably adequate.

On the other hand, the defects in fetal development in the parathyroidectomized rats, as far as these may be judged from chemical analysis of the newborn, were somewhat aggravated by the calcium deficiency of diet no. 26.

Cox and Imboden ('36 b) found that after several reproductive cycles, diet no. 16 (Ca 1.225%, P 0.245%) produced a lowered ash content in the offspring and a condition comparable to congenital rickets. These findings were confirmed in the present study, not only for second and subsequent litters, but for first litters as well. The newborn rats in this group yielded the lowest average values for total solids, calcium, phosphorus and Ca/P ratio.

The properties of diet no. 16 in offsetting the effects of parathyroid deficiency in the pregnant rat have been described in other connections (Bodansky and Duff, '41 a, b). Hypocalcemia, the associated tetany, and the high rates of maternal and fetal mortality are abolished. The bones of the parathyroidectomized rats undergo changes in weight comparable to those in the normal rats on the same diet, suggesting equivalent rates of demineralization and calcification. Finally, the composition of the young from parathyroparvic mothers fed diet no. 16 is the same as that for the corresponding controls (table 1). Fetal storage of calcium and phosphorus is practically identical.

The results may be interpreted as signifying that on this diet the mineral metabolism on the fetal side of the placental barrier is essentially the same in both groups of rats. Maternal parathyroid function, of primary importance when other diets are fed, is evidently not a significant factor in fetal development under these circumstances.

Diet no. 19 (Ca 1.225%, P 1.225%), containing the same high percentage of calcium as diet no. 16, but sufficiently more phosphorus to bring the Ca/P ratio down to a value of 1, differed from diet no. 16 in enabling the deposition of normal amounts of calcium and phosphorus in the fetuses of the control rats. The pups of the parathyroidectomized group were low in body weight, per cent ash, calcium, and in the Ca/P ratio of the ash. Total fetal storage of calcium and phosphorus was less than in the corresponding group on diet no. 16.

In normal rats fed diet no. 8 (Ca 0.49%, P 0.735%), fetal development was substantially the same as on diets 7 and 27. However, rats fed diet no. 12 (Ca 0.735%, P 0.49%) produced young which were somewhat heavier than the offspring in other diet groups and contained higher percentages of solids, ash, calcium, and phosphorus.

The pups from the parathyroidectomized rats on diets 8 and 12 were inferior to the controls on the corresponding diets. In relation to pups from other groups of parathyroidectomized rats they were somewhat superior. On diet no. 12, fetal storage of calcium and phosphorus attained values which were higher than those for any other group of parathyroidectomized animals.

Shelling ('32), and Richter and Eckert ('37), have shown that parathyroidectomized rats eat freely of diets rich in calcium and refuse diets rich in phosphorus, an observation confirmed by the present study. Diet no. 16, overabundant in calcium but low in phosphorus, was preferred to other diets by parathyroidectomized rats. Diet no. 19 was rejected by a few, but not by all animals, while diet no. 10 (Ca 0.49%, P 2.45%) was refused almost invariably. As a rule, the parathyroidectomized rats offered diet no. 10 lost weight rapidly

and, unless the ration was changed, death resulted from starvation.

Owing to these circumstances it was almost impossible to carry a parathyroidectomized rat through a successful pregnancy. Out of a group of more than twelve animals this was achieved only once. During the first month on diet no. 10 this animal lost nearly 80 gm. in weight. As a result of the third mating it became pregnant and regained approximately 50 gm. in weight during the period of gestation, which was somewhat prolonged. Tetany developed at term. Nine live pups were removed from the uterus. Analysis of maternal blood collected from the carotid artery during delivery gave the following results: serum Ca 3.2 mg. per 100 cc., P 5.25 mg. per 100 cc. The low weights of the pups and other data are given in table 1.

With most diets appetite was not significantly altered because of lack of parathyroid function. In general, the parathyroidectomized rat and its corresponding unoperated control consumed approximately the same quantities of food during pregnancy, as shown by the data in table 2. In some instances more food was consumed; in others somewhat less. Parathyroidectomized rat no. 526, fed diet no. 7 of Cox and Imboden, though consuming more food than rat no. 525, its control, nevertheless utilized for fetal development only about half of the calcium and phosphorus stored by the fetuses of the unoperated controls. This relationship held, not only for diet no. 7, but for certain other diets, as brought out by the data in table 2, which were typical of a larger number of similar balance experiments. These data provide conclusive evidence that on a given diet (7, 19, 26, 27) the differences in fetal mineral metabolism between normal and parathyroid-deficient rats cannot be attributed to quantitative differences in food consumption.

It is instructive to compare the utilization of calcium and phosphorus at various levels of intake. The data in table 2 for diets 26 and 27 reflect either a greater mineral depletion from the maternal organism, or a more economical utilization

TABLE 2  
Showing that differences in the fetal storage of calcium and phosphorus between normal and parathyroidectomized rats fed a given diet were not due to differences in food consumption

RAT SERIAL NO.	DIET NO.	NORMAL CONTROLS (N-C) OR PARATHYROIDECTOMIZED (P-D)	INCREASE IN WEIGHT DURING PREGNANCY	FOOD CONSUMED gm.	CONSUMPTION OF Ca P gm.	NUMBER OF PUPS	WEIGHT OF LITTER gm.	FETAL STORAGE		
								Calcium Amount	Part of consumption	Phosphorus Amount
525	7	N-C	178-240	21.9	1.073	12	48.4	117.7	11.0	144.0
526		P-D	208-247	26.0	1.274	10	33.4	52.7	4.1	79.3
420		N-C	178-255	28.9	1.416	7	42.6	122.1	8.5	135.9
421	7	N-C	185-293	32.6	1.607	14	69.9	182.9	11.4	229.1
419		P-D	212-295	28.2	1.382	12	47.4	64.6	4.7	128.4
375		N-C	284-370	23.3	1.142	11	65.8	178.8	15.6	205.9
376	7	N-C	236-347	24.6	1.205	9	47.4	107.9	9.0	134.5
378		P-D	222-290	22.6	1.107	8	31.0	78.8	7.1	99.5
397		P-D	177-224	21.4	1.049	10	36.2	64.3	6.1	103.5
387	27	N-C	277-306	23.3	0.287	9	51.9	126.8	42.7	138.1
383		P-D	208-260	20.4	0.254	9	31.0	51.8	20.8	92.0
393		N-C	253-350	27.7	0.388	12	64.6	172.4	51.0	177.5
414	27	N-C	196-280	26.4	0.322	11	54.3	133.2	41.4	176.4
408		P-D	186-222	32.3	0.394	12	25.6	32.2	8.1	68.8
537 F <sub>2</sub>		N-C	213-284	24.4	0.041	10	52.1	119.5	292.0	152.6
538	26	P-D	227-244	26.2	0.044	8	14.0	19.0	43.2	36.0
541		N-C	170-250	18.0	0.031	0.442	9	49.0	125.7	405.0
581		P-D	221-255	23.4	0.040	0.574	8	33.1	57.7	144.0
350	16	N-C	200-243	19.6	2.460	6	36.0	75.3	3.1	105.5
349		P-D	186-257	18.4	2.250	8	42.8	70.3	3.1	103.5
567	19	N-C	160-248	21.7	2.660	10	49.7	112.8	4.2	139.8
566		P-D	212-255	23.3	2.860	9	28.9	66.4	2.3	92.0

of the calcium and phosphorus supplied in the rations. Studies now in progress indicate that both factors participate in varying degree, depending upon the composition of the maternal diet. When the diet is almost devoid of calcium, as is the case with diet no. 26, most of the calcium for fetal development is derived from the maternal bones. Very little is lost by excretion. A balance experiment performed on rat 537 during the first reproductive cycle gave the following results: calcium consumed during the last 18 days of pregnancy, 30 mg.; calcium excreted in feces and urine during the same period, 45 mg.; calcium contained in newborn pups, 116.4 mg.—equivalent to 387% of the calcium consumed during the last 18 days of pregnancy, or an estimated 320% of the amount consumed during the entire gestation period. The low excretion of calcium in rat 537 may be contrasted with an excretion of 639 mg. in another rat (no. 529), fed diet no. 7. On this diet, the rat consumed 900 mg. of calcium during the last 15 days of pregnancy. Fetal storage of calcium amounted to 109 mg. leaving a positive balance of 152 mg., representing the storage of calcium in the maternal organism.

In the parathyroidectomized rats the percentage utilization of dietary calcium for fetal development on a given diet was invariably much lower than in the corresponding controls, with the exception of those on diet 16. As shown by the data in table 2, the utilization, 3.14%, was identical for the parathyroidectomized rat and for its unoperated control.

Under conditions of extreme deficiency, as with diet no. 26, even the parathyroidectomized rats seemingly possessed some ability to mobilize calcium from the maternal reserves. Such mobilization was much less responsive to the needs of the organism and more variable than was the mobilization of bone salts in the animal with intact parathyroid function.

The data in table 3 for rats 421 (diet no. 7), 392 (diet no. 27) and 539 (diet no. 26), were selected for the reason that in each of these animals fetal storage of calcium attained the highest level for its respective diet group. A comparison of the three figures, 182.9 mg., 184.0 mg., and 182.8 mg., brings

out the remarkable fact that the fetal storage of calcium was nearly identical in these animals in spite of enormous differences in calcium intake during pregnancy. Here is conclusive evidence that in a young rat (no. 539, age 95 days) fed a diet nearly devoid of calcium, the requirements for fetal development were met from its own reserves. During pregnancy this rat gained 85 gm. in weight, of which 68.9 gm. represented the weight of the pups. The average birth weight, 5.75 gm., was

TABLE 3

*Data selected to show that the fetal storage of calcium and phosphorus may attain approximately the same high levels in spite of large differences in the maternal intake of these elements during pregnancy*

Rat serial number	421	392 <sup>1</sup>	539
Diet number	7	27	26
Age at onset of pregnancy	95 days	175 days	95 days
Change in weight during pregnancy, in gm.	185-285	250-338	225-310
Average weight of pups at birth, in gm.	5.0	6.3	5.7
Number of pups in litter	14	12	12
Weight of litter, in gm.	70.0	75.0	68.4
Consumption during last 18 days of pregnancy			
Calcium, in gm.	1.600	0.408	0.037
Phosphorus, in gm.	1.600	0.819	0.536
Fetal storage			
Calcium, in mg.	182.9	184.0	182.8
Per cent of consumption	11.4	45.4	494.0
Phosphorus, in mg.	229.1	222.8	211.9
Per cent of consumption	14.3	27.2	39.5

<sup>1</sup> This was a second litter; 421 and 539 were first litters.

relatively high. The total calcium content of the pups, 182.8, was equivalent to 494% of the calcium consumed during the last 18 days of pregnancy. Assuming, on the basis of other observations with diet no. 26, an excretion of about 50 mg. of calcium during the same period, the total loss of this element from the maternal organism amounted to about 200 mg., or an estimated 10% of its original reserve.

These relations account for the normal fetal development in a first pregnancy and, frequently, in a second pregnancy,

as well, but as the maternal calcium reserve is reduced, further loss during subsequent pregnancies is minimized, usually because of reduced fertility (fewer pups per litter). However, occasionally, fetal development and calcification are defective. These untoward effects may be obviated largely by providing the mother rat with an adequate diet for about a week between pregnancies. Thus, in one experiment in which a rat was fed diet no. 26 over a period of 5 months, but with 1-week intervals between pregnancies on diet no. 7, the fifth litter turned out to be best, consisting of fourteen pups, weighing 75.6 gm. and containing 163 mg. of calcium and 202 mg. of phosphorus.

#### DISCUSSION AND EXTENSION OF DATA

Excluding the consequences of repeated pregnancies in the face of long-continued dietary deficiency, the fact is that in the rat, as in man, fetal development and calcification may be normal under conditions of extreme calcium privation to the mother. Such independence for the fetus is, therefore, in marked contrast to the well-recognized dependence, postnatally, of the growing organism on its calcium intake (Sherman and Booher, '31; Fairbanks and Mitchell, '36; Cox and Imboden, '36 b; Boelter and Greenberg, '41; and others). It would seem, then, that in spite of marked variations and abnormalities in the nutrition and metabolism of the maternal organism, the mineral metabolism of the fetus may remain undisturbed. This condition, governed by regulatory mechanisms, may be included, properly, in the category of phenomena which Cannon ('29) designated by the term "physiological homeostasis". Maintenance of physiological homeostasis in the fetus when the maternal diet is deficient in calcium depends principally on a combination of two factors, the existence in the maternal organisms of a mobilizable calcium reserve and adequate stimulation of parathyroid activity.

#### *Rachitogenic diet*

These regulatory mechanisms evidently fail when the diet is disproportionately high in calcium. The rachitogenic prop-

erties of diets with a high Ca/P ratio are now well recognized, Bethke, Kick and Wilder ('32) having shown that increasing the ratio from 1.0 to 5.0 causes in rats a progressive decrease in growth, bone ash, and percentage of inorganic phosphorus in the blood serum. Similar observations have been recorded by Brown and co-workers ('32), Shohl and Wolbach ('36), and others, while Cox and Imboden ('36 b) found that such diets produce defective fetal calcification, an observation which has been confirmed and amplified in the present study.

As has been brought out, diet no. 16 ( $\text{Ca}/\text{P} = 5$ ) promotes demineralization of the maternal skeleton, at the same time impairing fetal calcification. The data needed to explain the effects in the fetus on a quantitative basis are incomplete, but in anticipation of further investigation, their relation to the composition of the maternal blood, and, presumably, also to that of the fetal blood may be suggested. It is significant that in five out of six rats fed diet no. 16, the concentrations of inorganic phosphorus of the blood serum at term was 2.5 mg. per 100 cc., or less, values which are very low for the rat. The serum calcium averaged 11.54 mg. The  $\text{Ca} \times \text{P}$  product for the group averaged 21.9, and was the lowest encountered for any normal group of rats.

In this connection it will be recalled that Howland and Kramer ('22) brought out the relation of the  $\text{Ca} \times \text{P}$  product to calcification. They showed that in rickets the product was 35, or less, whereas normally the product was 40, or more. Logan ('40), in a review of the chemistry of calcification, notes that although the product can now be more accurately expressed in terms of  $\text{Ca}^{++} \times \text{HPO}_4^-$ , the original formulation of Howland and Kramer still retains its practical value.

If one resorts to this formula in order to explain the defective fetal calcification in rats fed diet no. 16, he may encounter several objections, one being that the only available analyses were those made at term; another is that the data pertain to the maternal and not to the fetal blood. However, from other evidence (Collip, '27; Needham, '31; Mull, '36) it may be assumed that the concentrations of calcium and phosphorus

on the fetal side of the placental barrier, though somewhat higher than on the maternal side, run roughly parallel. Evidently, this relation prevails even when one or both constituents are abnormally low in the maternal circulation, as illustrated by the following data recorded by Maxwell ('34) in a case of osteomalacia:

Maternal blood during delivery:	Ca 5.4 mg., P 2.9 mg. per 100 cc.
Cord blood:	Ca 8.1 mg., P 4.2 mg. per 100 cc.

Viewed in this light and also from the standpoint of Howland and Kramer's ('22) concept, the low Ca  $\times$  P product in the rats fed diet no. 16 assumes significance. The product averaged 21.9, and in one instance was only 6.3, representing conditions which were incompatible with normal bone salt deposition. In rats fed other diets, the products were as follows (average values): diet no. 7, 42.5; diet no. 8, 53.4; diet no. 10, 49.1; diet no. 12, 52.9; diet no. 19, 42.6; diet no. 26, 31.9; diet no. 27, 47.0. These figures may be correlated with the finding that, out of all the diets employed in these studies, diet no. 16 stood out, alone, as the one which prevented normal fetal calcification in otherwise normal rats.

#### *Maternal parathyroid deficiency*

In parathyroidectomized rats, the principal mechanism for mobilizing calcium from the bones is abolished. Excretion of phosphorus is also impaired, as a consequence of which the concentration of this element in the blood tends to increase. Accordingly, even though the calcium is sharply reduced, the Ca  $\times$  P product is not invariably low.

Correlation of the product with fetal calcification is, therefore, not as consistent as in the unoperated controls. Thus, for the parathyroidectomized rats fed diet no. 27, the product based on average values for calcium and phosphorus was  $4.24 \times 10.76 = 45.7$ , a figure theoretically compatible with normal calcification, though, actually the fetal storage of calcium and phosphorus was deficient. The explanation for this paradox may lie in the observation of McLean, Barnes

and Hastings ('35) that parathyroid deficiency causes a sharp reduction in  $\text{Ca}^{++}$  ions, and the related demonstration by McLean and Hinrichs ('38) that increasing the phosphate concentration in the blood promotes the formation of a colloidal calcium phosphate at the expense of calcium ions and calcium bound to protein.

For other diets, the  $\text{Ca} \times \text{P}$  products in the parathyroidectomized rats were as follows (average values): diet no. 7, 36.4; diet no. 8, 27.2; diet no. 10, 16.8; diet no. 16, 21.8; diet no. 19, 32.1; diet no. 26, 22.1.

On the basis of the analytical data presented in this and previous papers it could be concluded that the fetal growth and calcification depend not so much on the calcium and phosphorus contained in the diet as on the levels of calcium and phosphorus maintained in the blood, normal levels of both in the mother and fetus promoting normal calcification, low levels of either preventing such calcification and inhibiting fetal growth generally.

Final proof of this would require analysis of the fetal blood and other data which, owing to technical limitations, are as yet unavailable. However, the indirect evidence is significant. On the basis of Kozelka, Hart and Bohstedt's report ('33) that in parathyroidectomized pregnant dogs the hypocalcemia and associated symptoms may be relieved by the administration of viosterol, 400 units of this vitamin were given daily during pregnancy to rats fed diet no. 7. While this did not produce a uniform effect on the serum calcium at term, the average for the group ( $5.4 \pm 1.8$  mg.) was somewhat elevated. This elevation, though moderate, was accompanied by definite improvement in fetal development. Analysis of thirteen litters from parathyroprevic mothers yielded the following average values and standard deviations from the mean: number of pups per litter  $10.1 \pm 1.7$ ; birth weight  $4.1 \pm 0.7$ ; ash  $1.64 \pm 0.12\%$ ; calcium  $0.216 \pm 0.031\%$ ; phosphorus  $0.295 \pm 0.022\%$ ; total fetal storage of calcium  $88 \pm 22$  mg.; total fetal storage of phosphorus,  $120 \pm 29.8$  mg.

*Effect of aluminum*

More remarkable than the effects produced by viosterol in parathyroidectomized pregnant rats were those obtained by incorporating 4% of aluminum acetate in diet no. 7. Jones ('36) found that aluminum acetate, or sulfate, protects rats from the symptoms which usually follow parathyroidectomy, the effect of the aluminum being related to reduction of the serum phosphorus and increase in serum calcium. Our results for pregnant rats agree with Jones' data for non-pregnant rats, as shown by the following values (averages and standard deviations) for eight parathyroidectomized animals at term: serum calcium  $7.68 \pm 1.90$ ; phosphorus  $4.23 \pm 2.41$ . The serum calcium was considerably above, and the phosphorus considerably below, the corresponding values previously established for pregnant parathyroidectomized rats fed diet no. 7 (Ca  $4.65 \pm 1.06$ ; P  $7.81 \pm 4.77$ ; Bodansky and Duff, '41 a).

Analysis of the eight litters from these animals yielded the following average values: pups per litter,  $10.3 \pm 3.4$ ; birth weight,  $4.5 \pm 0.7$ ; ash,  $1.59 \pm 0.115\%$ ; calcium,  $0.203 \pm 0.026\%$ ; phosphorus,  $0.254 \pm 0.023\%$ ; total fetal storage of calcium,  $93.8 \pm 40$  mg.; total fetal storage of phosphorus,  $126.1 \pm 46.0$ .

## SUMMARY AND CONCLUSIONS

1. In rats with intact parathyroids fetal growth and storage of calcium and phosphorus may show little difference in spite of large differences in the maternal intake of these elements. A diet nearly devoid of calcium does not necessarily prevent normal fetal growth or alter the mineral composition of the offspring.
2. However, if the maternal diet contains an overabundance of calcium, so that the Ca/P ratio is abnormally high (diet no. 16 of Cox and Imboden, Ca 1.225%, P 0.245%, Ca/P ratio = 5), the deposition of bone salts in the embryo is definitely impaired.
3. Normal development of the fetus, including growth and calcification, depends on the parathyroid function of the

maternal organism. Lack of parathyroid secretion during pregnancy disturbs the mineral metabolism, not only on the maternal side, but also on the fetal side of the placental barrier.

4. The dominance of maternal parathyroid function as a factor in fetal development was abolished in rats fed rachitogenic diet no. 16 of Cox and Imboden. On this diet, parathyroidectomized and normal rats produced young which did not differ significantly in composition.

5. On a given diet, differences in the fetal storage of calcium and phosphorus between normal and parathyroidectomized rats were not due to differences in the amounts of food consumed during the period of gestation.

6. Administration of viosterol to parathyroidectomized rats fed certain diets produced a moderate increase in the average concentration of serum calcium and definite improvement in average fetal growth and fetal storage of calcium and phosphorus.

7. An even more conspicuous effect was obtained by incorporating 4% of aluminum acetate in the diet of the pregnant parathyroidectomized rats. Associated with the reduction of the serum inorganic phosphorus and increase in the serum calcium there was marked improvement in fetal growth and in fetal storage of calcium and phosphorus.

8. It is concluded that the development of the fetus and its ability to store calcium and phosphorus depend on the maintenance of suitable concentrations of calcium and phosphorus in the blood on the maternal side, and, presumably, also on the fetal side, of the placental barrier.

#### LITERATURE CITED

- BETHKE, R. M., C. H. KICK AND W. WILDER 1932 The effect of the calcium-phosphorus relationship on growth, calcification, and blood composition of the rat. *J. Biol. Chem.*, vol. 98, p. 389.
- BODANSKY, M., AND V. B. DUFF 1941 a Effects of parathyroid deficiency and calcium and phosphorus of the diet on pregnant rats. *J. Nutrition*, vol. 21, p. 179.  
— 1941 b Relation of parathyroid function and diet to the mineral composition of the bones in rats at the conclusion of pregnancy. *J. Nutrition*, vol. 21, p. 235.

- BOELTER, M. D. D., AND D. M. GREENBERG 1941 Severe calcium deficiency in growing rats. *J. Nutrition*, vol. 21, p. 75.
- BROWN, H. B., A. T. SHOHL, E. E. CHAPMAN, C. S. ROSE AND E. M. SAURWEIN 1932 Rickets in rats. XIII. The effect of various levels and ratios of calcium to phosphorus in the diet upon the production of rickets. *J. Biol. Chem.*, vol. 98, p. 207.
- CANNON, W. B. 1929 Organization for physiological homeostasis. *Physiol. Rev.*, vol. 9, p. 399.
- COLLIP, J. B. 1927 Some observations on the composition of the blood and tissues of the foetal calf. *Trans. Roy. Soc. of Canada*, vol. 21, p. 147.
- COX, W. M., JR., AND M. IMBODEN 1936 a The role of calcium and phosphorus in determining reproductive success. *J. Nutrition*, vol. 11, p. 147.
- 1936 b The mineral composition of young rats. *J. Nutrition*, vol. 11, p. 177.
- FAIRBANKS, B. W., AND H. H. MITCHELL 1936 The relation between calcium retention and the store of calcium in the body, with particular reference to the determination of calcium requirements. *J. Nutrition*, vol. 11, p. 551.
- HOWLAND, J., AND B. KRAMER 1922 Factors concerned in the calcification of bone. *Trans. Am. Ped. Soc.*, vol. 34, p. 204.
- JONES, J. H. 1936 The relation of serum phosphates to parathyroid tetany. *J. Biol. Chem.*, vol. 115, p. 371.
- KOZELKA, F. L., E. B. HART AND G. BOHSTEDT 1933 Growth, reproduction, and lactation in the absence of the parathyroid glands. *J. Biol. Chem.*, vol. 100, p. 715.
- LOGAN, M. A. 1940 Recent advances in the chemistry of calcification. *Physiol. Rev.*, vol. 20, p. 522.
- MCCRUDDEN, F. H. 1909-1910 The quantitative separation of calcium and magnesium in the presence of phosphates and small amounts of iron devised especially for the analysis of foods, urine and feces. *J. Biol. Chem.*, vol. 7, p. 83.
- MCLEAN, F. C., B. W. BARNES AND A. B. HASTINGS 1935 The relation of the parathyroid hormone to the state of calcium in the blood. *Am. J. Physiol.*, vol. 113, p. 141.
- MCLEAN, F. C., AND M. A. HINRICHES 1938 The formation and behavior of colloidal calcium phosphate in the blood. *Am. J. Physiol.*, vol. 121, p. 580.
- MAXWELL, J. P. 1934 Osteomalacia and diet. *Nutrition Abstracts and Reviews*, vol. 4, p. 1.
- MULL, J. W. 1936 Variations in serum calcium and phosphorus during pregnancy. III. The effect on the fetal circulation. *J. Clin. Invest.*, vol. 15, p. 513.
- NEEDHAM, J. 1931 *Chemical Embryology*. The University Press, Cambridge, England, vol. 3, pp. 944, 1521, 1528.

- RICHTER, C. P., AND J. F. ECKERT 1937 Increased calcium appetite of parathyroidectomized rats. *Endocrinology*, vol. 21, p. 50.
- SHELLING, D. H. 1932 Calcium and phosphorus studies. I. The effect of calcium and phosphorus of the diet on tetany, serum calcium, and food intake of parathyroidectomized rats. *J. Biol. Chem.*, vol. 96, p. 195.
- SHERMAN, H. C., AND L. E. BOOHER 1931 The calcium content of the body in relation to that of the food. *J. Biol. Chem.*, vol. 93, p. 93.
- SHOHL, A. T., AND F. G. PEDLEY 1922 A rapid and accurate method for calcium in urine. *J. Biol. Chem.*, vol. 50, p. 537.
- SHOHL, A. T., AND H. B. BROWN 1932 In J. P. Peters and D. D. Van Slyke's *Quantitative Clinical Chemistry*, Williams and Wilkins, Baltimore, p. 869.
- SHOHL, A. T., AND S. B. WOLBACH 1936 Rickets in rats. XV. The effect of low calcium-high phosphorus diets at various levels and ratios upon the production of rickets and tetany. *J. Nutrition*, vol. 11, p. 275.



## THE GOITROGENICITY OF SOYBEANS<sup>1</sup>

H. S. WILGUS, JR., F. X. GASSNER, A. R. PATTON<sup>2</sup> AND R. G. GUSTAVSON

*Colorado State College Experiment Station, Fort Collins, and  
University of Colorado, Boulder*

(Received for publication January 20, 1941)

In 1938, Patton, Wilgus, and Harshfield ('39) succeeded in producing goiter experimentally in chickens. The ration used in this work contained 25% of soybean oil meal as the chief protein supplement. About that time, Sharpless ('38) reported that a diet containing 75% of raw soybean flour induced in the thyroid of young rats an enlargement of three to five times normal, thus confirming the earlier evidence of McCarrison ('34) that soybeans would produce an enlargement of the thyroid of rats. It therefore appeared possible that the goiter observed in chickens was caused by the amount of soybean oil meal used, which was considerably in excess of that customarily employed. Hence, a study was undertaken to ascertain whether soybeans and soybean oil meal are goitrogenic to poultry and whether goiter can be produced without their presence in the diet.

### EXPERIMENTAL

Single-comb white Leghorn chicks of a closely inbred strain were used in the experiments described in this paper. The chicks were hatched from hens receiving diets with known concentrations of iodine. The white rats used in one experiment were likewise closely inbred.

All experiments with chicks were conducted in battery brooders. Temperature in the battery room was maintained at 70° F. in winter and rarely went over 85° F. in summer.

<sup>1</sup> This is paper 116, Scientific Journal Series, Colorado Experiment Station.

<sup>2</sup> Now head, Chemistry Department, Montana Agricultural Experiment Station, Bozeman, Montana.

Lights were used to maintain a 14-hour day. The chicks were evenly distributed in the various pens. They were weighed individually each week. The rats were confined in individual cages with wire screen floors. At the end of the experimental period in experiment 1, representative chicks and rats were selected from each pen according to sex, weight, and condition. These animals were killed, and their thyroid glands were removed. The chick glands were trimmed and weighed immediately. Owing to the difficulty of accurate and quantitative removal, the rat glands were not weighed. The glands were fixed in Bouin's solution, sectioned, and stained by a special method to be described elsewhere.

The special staining method was developed by a series of modifications of Mallory's connective tissue stains, following the observation by one of us (F.X.G.) that the colloid of the thyroid follicles may be stained selectively red or blue, depending upon its density and possibly upon its high or low iodine content. A standard was evolved on the basis of which thyroid histology could be more closely correlated with thyroid function and with the nutritional status of the experimental animals kept on various diets. A systematic study of thyroid glands showed that they could be readily classified in ten distinct groups. Each group was assigned a mathematical score. Scores from 1 to 5 marked the range of goiter observed from a mild hyperplasia and hypertrophy to the diffuse type of severe struma. In the other direction the scores of minus 1 to minus 4 stood for thyroid glands of diminished activity ranging from slight inactivity to colloid goiter. In the latter group fall most of the glands of birds which received large amounts of iodine supplementary to their rations.

Standard errors<sup>3</sup> were calculated for body weight, thyroid weight, and histological score. In experiments 2 and 3, all surviving chicks were sacrificed for thyroid glands. The thy-

<sup>3</sup> Standard errors were calculated as follows:  $S.E. = \sqrt{\frac{\sum W^2 - \bar{W}^2}{N(N-1)}}$

The error of the average body weight for both sexes was calculated by the formula:  $\sqrt{\frac{(S.E._1)^2 N_1 + (S.E._2)^2 N_2}{N_1 + N_2}}$

roid weight is expressed as milligrams of fresh thyroid per 100 gm. of body weight (Remington and Levine, '36). Since there was observed to be no relationship between body weight and goitrogenicity of the ration, growth data are omitted from the tables.

Iodine additions to the ration were made in the form of dilute alkaline, alcoholic solutions of KI. The amount of iodine was ascertained in each ration by a modification of the von Kolnitz and Remington method (Gassner, '40). Three experiments are reported here.

The basal rations used in these experiments were as follows:

	EXP. 1	EXP. 2 & 3
Ground yellow corn	51.5	54.0
Dried yeast residuals	5.0	7.5
Steamed bone meal	2.5	2.5
Vitamin D preparation <sup>4</sup>	0.05	0.05
Manganese sulphate	0.015	0.015
Sodium chloride	1.0	1.0
Total	60.065	65.065

The essential variables in each experiment are given in each table of results.

In experiment 1, eight pens, each containing twenty-five 1-day-old chicks, and a similar number of lots, each containing four male rats 28 days of age, were used. The chick experiment was conducted for 6 weeks and the rat experiment for 8 weeks. The following supplements were added to the basal ration: 35% of soybeans, ground fresh daily, or autoclaved; or 30% of expeller process soybean oil meal; or 28.7% of solvent process soybean oil meal; or 14% of casein and 1.5% of yeast. The supplements used in each ration are indicated in table 1. Five per cent of alfalfa leaf meal was added to each ration except in pen 6 in which it was replaced by 2½% each of yeast and starch. Corn starch was used to make a total of

<sup>4</sup>This was a solution of vitamin D containing 1,000 A.O.A.C. units per gram, prepared by diluting in Wesson oil a solution containing 50,000 units per gram. The active agent was designated "D-activated animal sterol in oil" by the manufacturer. We are indebted to Dr. J. J. Waddell of Du Pont de Nemours, Inc., for this material.

100 parts. The yeast variations were made in order that the calculated vitamin B<sub>2</sub> (G) content of all rations would be approximately the same. The soybean oil obtained by chloroform extraction and used in pen 8 replaced the same amount of corn starch. This extraction was done at room temperature, and the solvent was removed by low-vacuum distillation.

In experiment 2, thirteen pens, each containing twelve 10-day-old chicks, were used. These chicks had been depleted of their iodine reserves by holding them on the low-iodine basal ration from time of hatching. The duration of the experiment

TABLE I  
*Experiment 1*

PEN NO.	RATION	IODINE	CHICK THYROIDS			RAT THYROIDS	
			No.	Weight	Histology	No.	Histology <sup>1</sup>
		μg./kg.		mg./100 gm.			
1	Practical	313	6	7±0.7	0.0±0.0	2	0.0±0.0
2	35% soybeans	94	6	69±8.1	3.8±0.1	8	2.6±0.3
3	35% soybeans, autoclaved	94	6	22±3.8	2.2±0.2	2	1.8±0.3
4	28.7% soybean oil meal, solvent, A	81	7	62±17.2	3.7±0.2	2	2.5±0.5
5	30% soybean oil meal, expeller, B	81	7	46±10.4	2.5±0.3	2	2.5±0.0
6	Pen 5 ration but minus 5% alfalfa	25	6	149±24.0	5.3±0.2	5	3.7±0.3
7	14% casein	94	6	14±1.4	1.3±0.3	2	1.0±0.0
8	14% casein plus 6% chloroform extract	63	6	9±1.6	1.2±0.2	2	0.5±0.0

<sup>1</sup> According to the numerical scale devised. See text.

was 32 days. In experiment 3, nineteen pens were used. The first ten pens constituted a repetition of experiment 2 and the identical rations were used. These pens contained eleven 1-day-old chicks each. Each of the other pens contained twenty-five 1-day-old chicks. The duration of this experiment was 4 weeks. The additions of soybeans, soybean oil meal, and casein to the basal ration in experiments 2 and 3 were made in the same manner as in experiment 1 except in the case of pen 20. In this pen the soybean oil meal was doubled to 60%. This additional soybean oil meal replaced 25% of ground yellow corn and 5% of starch. A carotene solution was added to

approximate the vitamin A potency of the amount of yellow corn replaced. The soybean extracts replaced an equal amount of corn starch on the dry-weight basis, using casein as the protein supplement. The soybean residues acted as protein carriers. Corn starch was used as filler. In experiment 2, a control pen was included, the chicks in this pen being fed a practical starting mash recommended by this station for farm use.

The extractions of the soybeans were carried out at room temperature in a continuous extractor, acetone, ethanol, and ethyl ether being used in succession. It was necessary to heat the extracts to slightly above the boiling points of the solvents in order to remove all but a trace of the solvents. Final traces were removed by mixing with the ration and spreading the mixture out to dry.

Unpublished data have shown that thyroid weight per 100 gm. of body weight and thyroid histology in chicks on the same ration are essentially the same at 4 and 6 weeks of age. Since such data from duplicated pens in experiments 2 and 3 were in agreement they were combined and are so reported in table 2.

The intense goitrogenicity of soybeans is evident in all experiments (pens 2 and 10). The enlargement of the thyroid glands was from five to six times that obtained with the casein ration (pens 7, 11, and 21) at the same iodine level and was from eight to ten times normal (pens 1 and 9). Sharpless, Parsons and Prato ('39), using rats, obtained enlargement about four times normal. Mild goiter occurred on the casein ration, the production of goiter in chicks without the use of soybean oil meal being thus demonstrated. Expeller process soybean oil meal produced less severe goiter than did soybeans (pens 5 and 19), the enlargement being about four times that produced with casein. This indicates that the goitrogenic factor is heat-labile. Histologically, the rats in experiment 1 did not show as severe goiter as did the chicks, but the results with each were in essential agreement. It appears that the chick thyroid is more sensitive to goitrogenic rations than is that of the rat.

Attempts to extract the goitrogenic factor with various fat solvents met with no success. The soybean oil obtained by chloroform extraction in experiment 1 (pen 8) lowered the thyroid weight, but the microscopic structure in the chick thyroids was not altered. The severity of goiter in rats was slightly lessened by this oil. Further attempts to remove the

TABLE 2  
*Experiments 2 and 3*

EXP. NO.	PEN NO.	RATION	RATION IODINE	THYROID		
				Nos.	Weight mg./100 gm.	Histology <sup>1</sup>
2	9	Practical ration + iodine	18,000	6	12± 2.0	-1.0±0.0
2, 3	10	35% soybeans	38	12	100±18.2	3.3±0.16
2, 3	11	15% casein	25	19	16± 1.2	2.2±0.13
2, 3	12	3.9% acetone extract	31	18	16± 2.2	2.0±0.14
2, 3	13	1.2% alcohol extract	31	16	20± 2.1	2.0±0.17
2, 3	14	1.7% ether extract	31	13	19± 1.5	2.0±0.09
2, 3	15	28.0% acetone residue	18	10	79±13.8	3.6±0.15
2, 3	16	27.7% ethanol residue	13	16	139±23.9	3.1±0.31
2, 3	17	25.7% ether residue	25	13	77± 9.8	3.6±0.13
2, 3	18	Extracts + ether residue	31	14	65± 9.0	3.8±0.17
2, 3	19	30% soybean oil meal, expeller, C	19	37	59± 5.9	3.8±0.09
3	20	60% soybean oil meal, C	19	14	111±16.9	4.5±0.16
3	21	15% casein	15	23	21± 1.7	2.6±0.20
3	22	30% casein	12	19	19± 1.5	2.6±0.20
3	23	12% soybean oil meal, C, + 4% casein + 15% oat groats	19	9	74± 7.2	3.8±0.15
2, 3	24	Pen 19 ration + 5% alfalfa	38	17	44± 7.1	3.0±0.20
3	25	Pen 19 ration + iodine	56	6	26± 6.8	2.9±0.18
3	26	Pen 21 ration + 5% alfalfa	56	10	13± 1.3	2.3±0.23
3	27	Pen 21 ration + iodine	50	3	13± 2.2	0.5±0.20
2, 3	28	35% soybeans + iodine	18,000	15	12± 1.4	-0.3±0.13

<sup>1</sup> According to the numerical scale devised. See text.

goitrogenic factor by successive extractions with acetone, ethanol, and ethyl ether in experiments 2 and 3 failed to show any activity in the extracts (pens 11 to 14). The residues from the acetone and ether treatments (pens 15 and 17) differed only insignificantly in goitrogenicity from the original soybeans. The residue from the ethanol treatment (pen 16) produced significantly heavier goiters than did the other two

residues. This change in weight was not substantiated by the thyroid histology. It is of interest to note that all three extracts in experiments 2 and 3 significantly increased body weight.

The goitrogenic factor is somewhat heat-labile. Autoclaving the soybeans in experiment 1 caused a marked reduction in goitrogenicity. The expeller process oil meal was less goitrogenic than the unroasted solvent-process meal (pens 5 and 4). The latter, which is subjected to very mild heat in the extraction process, resembled the raw soybeans in ability to produce severe goiter. This has been confirmed in later work. Soybean oil meal prepared by the expeller process is subjected to considerable heat, whereas that prepared by the solvent process may have had much less heat treatment unless this is given as an additional step. For further details on this subject, reference may be made to the paper of Hayward ('36).

The data in table 2 (pens 19 and 20) demonstrate that the goitrogenic effect of soybean oil meal is quantitative at high levels. The histological examination of goiters produced by the ration containing 30% of soybean oil meal showed hypertrophy and diffuse hyperplasia of the follicular epithelium of such an extreme severity that doubling the amount of soybean oil meal to 60% could only slightly increase the degree of goiter, although it did practically double the parenchyma of the glands. The stroma of these glands was increased considerably, with a large number of parafollicular cells present. This effect is not due to an increase in protein level, since doubling the amount of casein had no effect (pens 21 and 22).

The effects of a modified goitrogenic ration (pen 23) indicate strongly that a ration containing 12% of soybean oil meal will produce as severe goiter as a ration containing 30%, even when casein is used as a protein supplement to equalize the protein level. Unpublished data indicate that oat groats have no effect on thyroid weight or histology in the chick.

An apparent antigoitrogenic action of alfalfa leaf meal was first noted in experiment 1 (pens 5 and 6). The alfalfa leaf meal contained a small amount of iodine, and the data from

pens 19 and 24-27 of experiments 2 and 3 show that the anti-goitrogenic effect was probably caused by this iodine. The marked effect of the addition of even a small amount of iodine in lessening the severity of goiter is in agreement with the observations of Remington and Levine ('36) on rats.

The addition of sufficient iodine (pen 28) will entirely overcome the effect of the goitrogenic factor in soybeans on thyroid weight and histology. This iodine addition did not improve growth. Other observations have shown that lower amounts of iodine are also preventive when soybean oil meal is used. Experiments to discover the critical level of iodine supplementation will be reported later.

#### DISCUSSION

Our results with chicks and rats corroborate the observations of Sharpless, Parsons and Prato ('39) with rats that soybeans are goitrogenic, that the goitrogenicity is partially inactivated by heat, and that iodine will counteract the effect on the thyroid gland. In addition, the goitrogenicity of commercial soybean oil meal is established.

The data reported in this paper do not support those of Sharpless and his co-workers regarding the solubility of this goitrogenic factor or factors. These workers, using thyroid weight and iodine content as criteria, found that acetone and ether extractions of the beans resulted in a significant lowering of the goitrogenicity of the residues. They did not test the extracts. In our experiments, using thyroid weight and histology as criteria, the residues from successive extractions of soybeans with acetone, ethanol, and ethyl ether were not significantly different in goitrogenicity from the original soybeans. Neither the extracts thus obtained nor a chloroform extract showed any goitrogenic activity. Special care was taken to guard against exposure of the soybeans and the residues to heat. The report of Sharpless and his co-workers does not mention such precautions.

As outlined previously, the soybeans were treated successively with acetone, ethanol, and ethyl ether. The failure of

the significantly larger goiters produced by the residue from ethanol treatment to show any significant differences histologically from those produced by the other two residues is noteworthy. If these results can be confirmed, it will seem possible either that there are two goitrogenic factors or that a single factor acts selectively and progressively upon two receptive components of the tissue of the thyroid gland, i.e., the follicular epithelium and the interstitial connective tissue.

Doubling the size of the goiters by increasing the soybean oil meal from 30% to 60% of the ration agrees with the similar findings of Sharpless and co-workers. The lack of response in the thyroid glands to increasing the protein content of the ration to the same extent with casein indicates that the increase in protein level in the ration is not responsible. It is difficult to explain why 12% of soybean oil meal was as effective as 30%.

The effectiveness of iodine in correcting goiter produced by soybeans in chicks without improving growth demonstrates that the goitrogenicity of soybeans does not explain the poor growth which the raw soybeans are generally known to afford. Soybean oil meal is one of the best protein supplements in animal and poultry feeding. Since no detrimental effects on growth or general well-being of growing chicks were noted in this study on rations containing as high as 30% of soybean oil meal and since the presence of a small amount of iodine corrects the goiter, it is the opinion of the authors that no changes in present recommendations on the practical use of soybean oil meal in chick rations are justified on the basis of this report.

#### SUMMARY

The observations of Sharpless, Parsons and Prato that soybeans are goitrogenic, that this goitrogenicity is partially inactivated by heat, and that iodine will counteract the effect on the thyroid gland have been corroborated. The goitrogenic factor in soybeans was not extracted by chloroform nor by successive treatment with acetone, ethanol, and ethyl ether.

This is not in accord with the report of Sharpless et al. This goitrogenic factor causes intense hyperplastic and hypertrophic changes in the thyroid glands of chicks and rats.

Soybean oil meal was found to be goitrogenic, but no detrimental effect other than that on the thyroid gland was noted in growing chickens.

Goiter was produced in chicks without the use of soybeans or soybean oil meal.<sup>5</sup>

#### LITERATURE CITED

- GASSNER, F. X. 1940 An improved iodine apparatus. *Indust. Eng. Chem.*, vol. 12, p. 120.
- HAYWARD, J. W. 1936 Utilization of soybeans. *The Grain and Feed Review*, September.
- MCCARRISON, ROBERT 1934 The goiterogenic action of soya-bean and ground-nut. *Ind. J. Med. Res.*, vol. 21, pp. 179-181.
- PATTON, A. R., H. S. WILGUS, JR., AND G. S. HARSHFIELD 1939 The production of goiter in chickens. *Science*, vol. 89, p. 162.
- REMINGTON, ROE E., AND HAROLD LEVINE 1936 Studies on the relation of diet to goiter. III. Further observations on a goitrogenic diet. *J. Nutrition*, vol. 11, pp. 343-357.
- SHARPLESS, G. R. 1938 A new goiter-producing diet for the rat. *Proc. Soc. Exp. Biol. and Med.*, vol. 38, pp. 166-168.
- SHARPLESS, G. R., JANICE PEARSONS AND GENEVA S. PRATO 1939 Production of goiter in rats with raw and with treated soybean flour. *J. Nutrition*, vol. 17, pp. 545-555.

<sup>5</sup> We are indebted to the Iodine Educational Bureau, Inc., New York, for the establishment of an Investigatorship at the Colorado State College Experiment Station, from which part of the expense of conducting this project was obtained.

## MAGNESIUM BALANCE STUDIES WITH INFANTS

CARROLL F. SHUKERS, ELIZABETH M. KNOTT AND  
FREDERIC W. SCHLUTZ

*Department of Pediatrics, University of Chicago, Illinois*

TWO FIGURES

(Received for publication January 16, 1941)

Magnesium is an essential element which exhibits a complex physiologic function. The major portion of the magnesium occurs in the skeleton where it constitutes one-fortieth to one-fiftieth of the calcium content. Magnesium is considerably more abundant than calcium in the soft tissues where, among other functions, it is an activator of the phosphatase and glycolytic enzyme systems and a depressor of muscular and neural irritability. For a discussion of magnesium metabolism the reader is referred to several recent reviews with extensive bibliographies (Schmidt and Greenberg, '35; Greenberg, '39; Duckworth, '39, and Stearns, '39).

Although it is improbable that the average mixed diet containing leafy vegetables is deficient in magnesium, the milk diets of infants during the first few months of life may contain less than optimal amounts of this element. Human milk has the low magnesium content of 3 to 6 mg. per cent whereas cow's milk contains 13 to 19 mg. per cent.

Two studies on magnesium balances in infants have been reported in the literature. Swanson ('32) presents data on three normal full-term infants who received breast milk and cow's milk with and without cod liver oil. With intakes of 5.4, 7.6, 11.2 and 10.4 mg. per kilogram of body weight per day there were retentions of 1.5, 1.5, 1.6 and 2.8 mg. per kilogram per day respectively. Urinary excretion of magnesium was small and the major portion of this element was lost through

the feces. Schlutz, Morse and Oldham ('33) published data from this laboratory on four infants followed through thirteen to eighteen metabolic balance periods. They had received powdered whole milk to which was added in some periods spinach, cellu-flour or artificial salt mixture. The magnesium intakes of the four infants averaged 15.5, 14.5, 14.2 and 13.7 mg. per kilogram per day with corresponding retentions of 1.2, 1.4, 0.8 and 0.2 mg. per kilogram. These investigators concluded, "In our experiments the retentions of magnesium have been small and very variable. . . . By far the greater amounts of magnesium were eliminated in the feces and in general the amount in the feces paralleled the total output of feces."

Further work by Swanson on the magnesium content of the fetus and on the composition of growth seems important. Iob and Swanson ('34) found 783 mg. of magnesium (286 mg. per kilogram of body weight) in one full-term fetus. This value is comparable to the data on the average magnesium content of forty-six fetuses reviewed by Macy and Hunscher ('34). Although there were some exceptions in the individual analyses, these authors found a fairly even increase in the total amount of magnesium retained from 83 mg. by the fourth lunar month to 784 mg. by the tenth lunar month. Givens and Macy ('33) had stated that the average magnesium content of the fetus at term was approximately 0.5 gm. In studying the composition of growth Swanson found that magnesium constituted 218 and 378 mg. per kilogram gain in weight when two infants received breast milk, but magnesium represented 802 and 582 mg. per kilogram of gain when the infants were on cow's milk formulas. Swanson stated that "the retentions of calcium and phosphorus in two full-term infants per kilogram gain in weight are much less than the amounts of these minerals found per kilogram of body weight for full-term infants at birth. In respect to magnesium it is quite the opposite in that the retentions per unit of growth exceed the concentrations per unit of body weight at birth." The above work would indicate the need for a constant adequate supply of magnesium for satisfactory growth.

The present study is reported in the hope that the data may be of value in determining the level of magnesium intake which will yield optimum retentions of this element for the infant. A series of metabolism studies have been conducted on each of nine male infants during the first 6 months of their lives. The infants were given milk formulas to which either corn syrup or honey was added with vitamin and mineral supplements. The infants remained in good health and the diets were adequate for satisfactory gains in weight. A more detailed description of the infants and technics is presented elsewhere (Knott, Shukers and Schlutz).

Each infant was studied for at least eight metabolism periods during each of which urine and feces were quantitatively collected for 5 consecutive days. Rest periods of 3 to 7 days intervened between the collection periods. After each change of diet an interval of several days was allowed to permit equilibrium before a new metabolism period was started. The urine was preserved by toluene and refrigeration, enough concentrated hydrochloric acid being added to make the urine acid to methyl red. Feces were marked off by carmine, preserved by acid alcohol (5% glacial acetic acid in 95% ethyl alcohol) and by refrigeration during the collection periods, and finally dried to constant weight before analysis. In addition to magnesium retentions, calcium and thiamine balances were determined during the same periods. Magnesium analyses were made according to the method of Fiske and Logan ('34) on filtrates from the calcium determinations.

During the eighty-two metabolic balance periods for which data appear in table 1, total magnesium intakes varied from 54.6 to 245.3 mg. per day with fecal losses amounting to 39.5 to 191.6 mg. per day, and urinary excretion of 1.0 to 21.6 mg. per day. Retentions were similarly variable within the limits of -23.5 and 105.9 mg. per day. The relation of the retention of magnesium to intake has been briefly summarized in table 2. During twenty-three periods with intakes of 11.8 to 15.0 mg. per kilogram per day, there were sixteen positive and seven negative balances with a mean positive retention of 1.11 mg.

*Daily magnesium balance in infants*

SUBJECT PERIOD	WT.	FOOD			URINARY MAGNESIUM IN MG. PER		FECAL MAGNESIUM IN MG. PER		RETENTION IN MG. PER			
		Milk	Carbo-hydrate	Vit. D	Magnesium in mg. per	day	kg.	day	kg.	day		
No. 1 John	4 kg. 5 6 7 8 9 10	E.L.A. E. " " " " " " " " " " " " " " "	Honey " " " " " " " " " " " " " " " " " "	170 " " " " " " " " " " " " " " " 680	86.1 96.1 89.7 93.9 97.5 83.3 92.8	15.4 16.3 14.4 14.2 14.3 11.8 13.0	2.3 9.9 7.1 9.5 10.3 8.0 7.2	0.4 1.7 1.1 1.4 1.5 1.3 1.0	56.0 61.0 60.0 60.7 74.6 68.5 87.6	9.5 10.4 9.6 10.2 10.9 9.7 12.3	27.9 25.2 22.7 23.7 12.6 5.8 - 1.8	5.0 4.3 3.6 3.6 1.8 0.8 - 0.2
	5 kg. 5.27 5.82 6.16 7.35 8.39	D. " " " " " " " " "	Honey Corn syrup Honey	170 154. 168. 168.	140. 26.5 27.3 26.5	8. 10.8 8.9 10.6	1.5 1.9 1.4 2.4	101.3 135.6 136.3 126.6	19.2 24.3 22.1 19.9	30.7 7.6 22.8 30.8	6.1 1.8 3.7 4.9 3.8	
	6 kg. 6.27 6.82 7.35 8.39	D. " " "	Honey	170	140. 154. 168. 168.	8. 10.8 10.6 15.3	1.5 1.9 2.4	101.3 135.6 136.3 128.1	19.2 24.3 22.1 20.0	30.7 7.6 22.8 24.6	6.1 1.8 3.7 4.9 3.8	
	7 kg. 7.64 8.25 8.78 9.35 10.35 11.94	E. " " " " " " " " " " " " " " "	Honey	0	75.0 73.1 80.0 79.2 79.7 79.7	20.6 18.7 18.8 17.6 16.7 15.8	5.2 5.8 4.9 5.7 5.3 4.7	1.4 1.5 1.1 1.3 1.1 0.9	59.8 79.3 55.8 57.3 64.4 75.7	16.4 20.3 13.1 12.7 13.5 15.0	10. - 11. 19.3 21.9 10.0 - 0.7	2.7 - 2.8 4.5 4.9 2.1 - 0.1
	8 kg. 8.64 9.30 9.45 9.52 9.78 10.04 11.94	E. " " " " " " " " " " " " " " " " " "	Honey	680	137.9 139.2 147.8 159.9	24.8 24.0 23.3 24.2	8.1 5.8 2.9 8.3	1.4 1.0 0.5 1.3	74.0 115.2 75.4 123.7	13.3 19.9 11.9 18.7	56.8 18.2 69.5 27.9	10.2 3.1 11.0 4.2
	9 kg. 8.96 9.45 9.52 9.78 10.04 11.94	D. " " " " " " " " " " " " " " "	Honey	170	173.6	25.6	8.4	1.2	83.6	12.3	81.6	12.0
	10 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	11 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	D. " " " " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	12 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	13 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	14 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	15 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	16 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	17 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	18 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	19 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	20 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	21 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	22 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	23 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	24 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	25 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	26 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	27 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	28 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	29 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 118.8 147.2 132.0 111.9	26.3 34.7 26.0 24.6 25.4 23.2 57.7	63.0 - 23.5 7.8 7.1 - 12.5 15.2 57.7	17.1 - 5.9 1.8 1.5 - 2.5 2.7 9.5
	30 kg. 9.69 9.99 9.45 9.52 9.78 10.04 11.94	Skim D. " " " " " " " " " " " " " " "	Honey	170	169.5 126. 140. 140. 154. 165. 168.	45.9 31.6 31.5 29.0 30.2 29.6 28.6	9.6 10.9 16.4 14.1 19.3 20.8 21.6	2.6 2.7 3.6 2.9 3.8 3.6 3.2	96.9 138.6 115.8 1			

per kilogram per day. Intakes of 15 to 20 mg. per kilogram occurred in ten periods, three of which were negative. The mean positive balance was 2.14 mg. per kilogram per day. There was one negative balance during seven periods on intakes of 20 to 25 mg. per kilogram which exhibited a mean positive balance of 3.99 mg. per kilogram. A mean positive balance of 4.16 mg. per kilogram per day was found in twenty-seven periods, two of which were negative, with intakes of

TABLE 2  
*Relation of retention of magnesium to level of intake*

INTAKE mg./kg./day		NO. mg./kg./day	MEAN RETENTION	TOTAL	MEAN RETENTION	NO.	MEAN RETENTION
Less than 15	Positive	16	2.06	23	1.11	33	1.42
	Negative	7	-1.06				
15-20	Positive	7	3.51	10	2.14		
	Negative	3	-1.07				
20-25	Positive	6	5.27	7	3.99	44	3.96
	Negative	1	-3.70				
25-30	Positive	25	4.69	27	4.16		
	Negative	2	-2.45				
30-35	Positive	6	7.60	10	3.42		
	Negative	4	-2.85				
More than 35	Positive	5	14.92	5	14.92		
	Negative	0					

25 to 30 mg. per kilogram. At levels of 30 to 35 mg. per kilogram there were six positive and four negative balances with a mean retention of 3.42 mg. per kilogram per day. Five positive balances with a mean of 14.92 mg. per kilogram were found in the five periods with intakes over 35 mg. per kilogram per day. In brief, thirty-three periods at levels of intake under 20 mg. per kilogram per day exhibited a mean retention of 1.42 mg. per kilogram, whereas forty-four periods with intakes between 20 and 35 mg. per kilogram gave a mean retention of 3.96 mg. per kilogram per day. Seven of the nine infants had one or more periods with negative balances. In several instances these negative balances occurred on rela-

tively high intakes but there were proportionately twice as many negative balances on intakes under 20 mg. per kilogram as occurred when more magnesium was ingested.

The fecal excretion of magnesium was equivalent to approximately 80% of the intake. In six periods the fecal losses exceeded the intake and only in nine periods was it less than 60% of the intake. This result is in accord with the findings of other investigators.

The urinary excretion of magnesium varied from 1.0 to 21.6 mg. per day with a mean of 10.5 mg. per day. In general the magnitude of both fecal and urinary losses varied directly with the intake although there were numerous exceptions.

Retentions were slightly greater when honey was the source of added carbohydrate than when corn syrup was used for this purpose. The results are summarized in table 3. With powdered half skim milk furnishing the magnesium, thirty periods on honey averaged a total retention of 24 mg. as compared to ten periods on corn syrup with an average retention of 16 mg. per day. When evaporated lactic acid milk was fed, thirteen honey periods had a mean total retention of 6 mg. in contrast to a mean retention of -0.4 mg. per day for twelve corn syrup periods.

In table 3 the results are also summarized in relation to the vitamin D intake. A range of vitamin D levels from 0 to 1000 international units per day apparently had no consistent effect upon the retention of magnesium by the different infants. No correlations were observed between the level of vitamin D administered and the amount of magnesium excreted in the feces.

Skim milk and protein milk were the source of magnesium in seven of the periods which are reported in table 3. Intakes were over 150 mg. per day and 30 mg. per kilogram. Retentions were of large magnitude constituting one-third or more of the intake. This may be interpreted as indicating either better absorption of magnesium from these types of milk supplemented with honey or decreased intestinal excretion during these periods.

TABLE 3  
*Magnesium balances in relation to vitamin D intake,  
sources of milk and carbohydrate*

TYPE OF MILK	ADDED CARBO-HYDRATE	VITA-MIN D INTAKE	NUMBER OF			DAILY MAGNESIUM BALANCE			
			Sub-jects	Periods	Negative balances	Intake total	Fecal per cent of intake	Urine total	Retention total
Skim	Honey	170	2	2	0	163	58	7	54
Powdered	Honey	170	1	1	0	150	59	10	52
protein	"	200	2	4	0	240	50	17	101
"	(total)		3	5	0	(mean) 222	52	16	92
Powdered	Honey	0	1	4	1	189	85	13	16
half-skim	"	100	1	4	0	144	74	11	29
skim	"	170	3	13	1	154	80	13	22
"	"	200	2	4	2	181	95	15	-3
"	"	680	1	5	0	152	62	7	51
	(total)		6	30	4	(mean) 161	78	12	24
	Corn syrup								
		170	3	10	2	150	80	14	16
"	(grand total)		7	40	6	(mean) 158	78	12	19
Evapo-rated	Honey	170	3	9	0	77	72	7	15
	"	680	1	1	1	93	94	7	-2
	(total)		4	10	1	(mean) 79	74	7	13
Evap.	Honey	0	1	3	1	76	87	5	6
lactic acid	"	170	3	3	1	74	82	6	1
	"	340	2	2	0	74	78	14	4
	"	500	2	2	0	80	69	16	9
	"	1000	1	3	1	80	82	5	10
	(total)		4	13	3	(mean) 77	80	8	6
	Corn syrup								
		170	2	5	3	81	83	13	5
		340	2	2	1	75	81	14	1
	"	500	2	4	2	107	86	16	-3
	"	680	1	1	1	96	93	11	-5
	(total)		2	12	7	(mean) 90	85	14	-0.4
"	(grand total)		4	25	10	(mean) 83	82	11	3
Evaporated milks	(grand total)		4	35	11	(mean) 82	80	10	6

When powdered half skim milk was a source of magnesium there were six negative balances out of forty periods. Mean intakes varied from 150 to 189 mg. (24 to 31 mg. per kilogram per day), fecal losses averaged 78% of the intake, and the mean retention was 3.72 mg. per kilogram per day.

When evaporated milk or evaporated lactic acid milk was the source of magnesium, with one exception mean intakes were less than 100 mg. per day. Eleven out of thirty-five balances were negative and the mean retention was 1.33 mg. per kilogram per day. The data do not indicate whether the type of milk or the level of intake is the controlling factor for these low retentions, but there is some evidence that the latter is more important. The summary of magnesium balances presented in figure 1 illustrates the probability that the magnesium content of the milk is a controlling factor in determining the magnitude of retentions.

It is interesting to contrast the behavior of subject no. 3 (Hal) on two types of milk. On powdered half-skim milk with an intake of 24.4 mg. per kilogram the retention was 8.1 mg. per kilogram, while evaporated lactic acid milk provided 18.8 mg. per kilogram but the retention was only 1.9 mg. per kilogram. The urinary excretion was unchanged. The fecal loss of magnesium was apparently the controlling factor in this instance since it constituted 62% of the intake on the first formula and 82% of the intake on the evaporated lactic acid milk formula. Fecal losses were also significant in period 9 for infants no. 8 and no. 9 (Guy and Geoffery) when 10 gm. of wheat germ were fed as a daily supplement. For these two periods the average daily magnesium of the feces represented 87 and 104% of the intake.

No correlation was found between the phosphorus loss in the feces and the fecal magnesium loss. There was also no apparent relation between the fecal phosphorus and the magnesium retention. The ratio of calcium to magnesium in the milk formulas varied from 12.5:1 to 7:1 with a mean of 10:1.

## MEAN MAGNESIUM BALANCES FOR INDIVIDUAL INFANTS

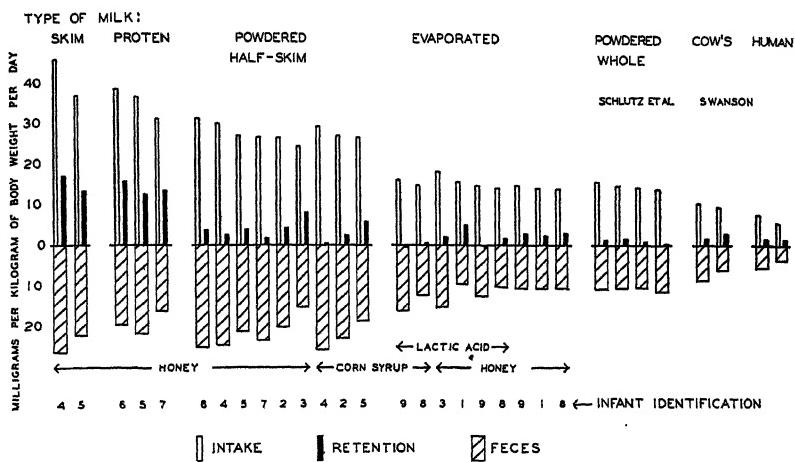


Figure 1

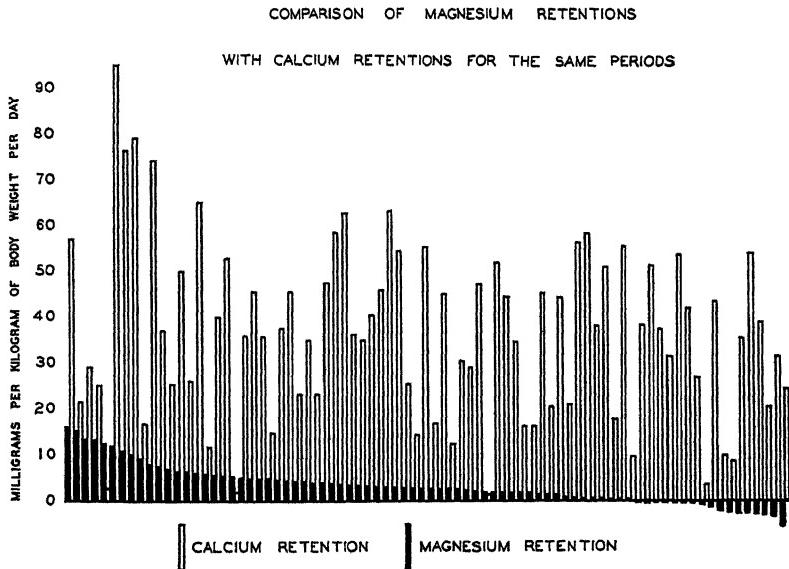


Figure 2

The ratio of calcium stored to magnesium retained was quite variable, but the mean of 10: 1 is comparable to the ratio found in pre-school children by Daniels and Everson ('36). Although there is a tendency for calcium retentions to become less as the magnesium retentions diminish (see fig. 2) there is no definite correlation between them. These findings support the conclusion that calcium, magnesium and phosphorus, although related in metabolism, respond differently to the diverse factors acting upon them.

Magnesium retentions of approximately the same magnitude on comparable intakes were reported in pre-school children by Daniels and Everson as were found in this study with infants. The statement is made by Schmidt and Greenberg that from one-fifth to one-half of the magnesium excreted by infants and adults appears in the urine. This was true for the pre-school children of Daniels and Everson, but the infants of this investigation as well as of other studies (Swanson; Schlutz, Morse and Oldham) have excreted only one-tenth to one-fifth of their total magnesium loss in the urine. Thus it appears from these data that the criterion for adequate magnesium intake by pre-school children, suggested by Daniels and Everson, i.e., urinary loss of more than 3.4 mg. per kilogram, does not hold for infants since urinary magnesium levels of less than 2 mg. per kilogram were associated with good retentions of magnesium.

The data of this study show that higher magnesium intakes tend to increase magnesium retentions, but the present state of our knowledge is not adequate to determine whether or not these higher retentions have a pertinent effect upon the infant's well-being. Further work is necessary to establish an optimal intake and also to prove the possible existence of a magnesium deficiency disease in infants. Further investigation is also necessary to determine which factors that influence magnesium metabolism are responsible for the extensive variability in results.

## SUMMARY

Infants from 2 to 6 months of age may be maintained in small but definite positive magnesium balance on diets containing 10 to 20 mg. per kilogram of body weight per day. Retentions<sup>a</sup> are of greater magnitude when the intake is more than 20 mg. per kilogram per day.

No definite relationship was noted: (1) between magnesium and calcium retentions although there was a slight tendency for the retention of both substances to vary in the same direction; (2) between the fecal phosphorus excretion and the magnesium content of the feces or the magnesium retention; and (3) between the level of vitamin D and the magnitude of magnesium retention.

Although the data are not conclusive, slightly better retentions were found when honey was the source of carbohydrate than were observed when corn syrup was added. The variations noted for the different types of milk used can probably be ascribed to the level of magnesium intake.

## LITERATURE CITED

- DANIELS, A. L., AND G. J. EVERSON 1936 A study of the magnesium needs of pre-school children. *J. Nutrition*, vol. 11, pp. 327-341.
- DUCKWORTH, J. 1939 Magnesium in animal nutrition. *Nutrition Absts. and Rev.*, vol. 8, pp. 841-860.
- FISKE, C., AND M. A. LOGAN 1934 In O. Folin, *Laboratory Manual of Biological Chemistry*, D. Appleton-Century Company, Inc., New York, 5th ed., p. 239.
- GIVENS, M. H., AND I. G. MACY 1933 The chemical composition of the human fetus. *J. Biol. Chem.*, vol. 102, pp. 7-17.
- GREENBERG, D. M. 1939 Mineral metabolism, magnesium. *Ann. Rev. Biochem.*, vol. 8, pp. 289-295.
- IOB, V., AND W. W. SWANSON 1934 Mineral growth of the human fetus. *Am. J. Dis. Child.*, vol. 47, pp. 302-306.
- KNOTT, E. M., C. F. SHUKERS AND F. W. SCHLUTZ 1941 Calcium balance studies with infants. *J. Pediatrics* (in press).
- MACY, I. G., AND H. A. HUNSCHER 1934 An evaluation of maternal nitrogen and mineral needs during embryonic and infant development. *Am. J. Obst. Gyneec.*, vol. 27, pp. 878-888.

- SCHLUTZ, F. W., M. MORSE AND H. OLDFHAM 1933 Vegetable feeding in the young infant. Influence on gastro-intestinal motility and mineral retention. Am. J. Dis. Child., vol. 46, pp. 757-774.
- SCHMIDT, C. L. A., AND D. M. GREENBERG 1935 Occurrence, transport and regulation of calcium, magnesium and phosphorus in the animal organism. Physiol. Rev., vol. 15, pp. 297-434.
- STEARNS, G. 1939 The mineral metabolism of normal infants. Physiol. Rev., vol. 19, pp. 415-488.
- SWANSON, W. W. 1932 The composition of growth. II. The full-term infant. Am. J. Dis. Child., vol. 43, pp. 10-18.

## NUTRITIVE PROPERTIES OF STEAM-RENDERED LARD AND HYDROGENATED COTTONSEED OIL

RALPH HOAGLAND AND GEORGE G. SNIDER

*Animal Nutrition Division, Bureau of Animal Industry, United States Department  
of Agriculture, Beltsville, Maryland*

(Received for publication December 2, 1940)

Lard and hydrogenated cottonseed oil are among the most important fats in use for shortening and cooking purposes in the United States. The relative merits of these fats for culinary purposes have been studied extensively but less attention has been given to their nutritive properties. The purpose of the experiments herein reported was to determine the relative growth-promoting properties and digestibility of steam-rendered lard and hydrogenated cottonseed oil when fed to young rats.

In a previous publication the writers ('40) discussed the results of earlier experiments concerning the nutritive properties of lard and hydrogenated cottonseed oil. They also reported the results of experiments which they had conducted with young rats with refined steam lard and a well-known brand of hydrogenated cottonseed oil. The two fats had approximately the same growth-promoting value when fat constituted 5% of the diet, but the lard was superior when fat constituted 30 or 55% of the diet. The refined lard was superior in digestibility to the hydrogenated cottonseed oil at each level of intake. The digestive coefficients for the two fats were 97.6 and 88.2% at the 5% level, 96.5 and 92.2% at the 30% level, and 95.1 and 93.7%, respectively, at the 55% level. The present experiments furnish additional information concerning the nutritive properties of lard and hydrogenated cottonseed oil and show the relative efficiency with which each fat was utilized at four levels of intake.

## MATERIAL AND METHODS

The lard and the hydrogenated cottonseed oil used in the present experiments were obtained from the same source. Only limited information concerning the manufacture of these products was supplied. The lard was steam rendered under 50 pounds pressure, settled, filtered, chilled, and packed in containers. It was not bleached. Refined cottonseed oil was hydrogenated to an iodine number between 70 and 75 and approximately 2% of fully hydrogenated oil was added. When

TABLE 1  
*Formulas for diets fed to rats*

DIET NO.	TYPE OF SHORTENING	FAT IN DIET	CASEIN	SALTS	DEX-TROSE	YEAST VITAMINS	COD LIVER OIL CONCENTRATE
1	No fat	0	18.6	4.0	75.4	2.0	100
2	Steam-rendered lard	5	20.0	4.3	68.7	2.0	100
3	Hydrogenated cotton-seed oil	5	20.0	4.3	68.7	2.0	100
4	Steam-rendered lard	15	23.0	5.0	54.5	2.5	100
5	Hydrogenated cotton-seed oil	15	23.0	5.0	54.5	2.5	100
6	Steam-rendered lard	30	27.8	5.8	33.4	3.0	100
7	Hydrogenated cotton-seed oil	30	27.8	5.8	33.4	3.0	100
8	Steam-rendered lard	54.2	34.8	7.3	0	3.7	100
9	Hydrogenated cotton-seed oil	54.2	34.8	7.3	0	3.7	100

received at the laboratory the fats were melted, transferred to glass jars, sealed, and stored at 4°C.

The formulas for the diets fed to the rats are shown in table 1. The casein, cod-liver oil concentrate, and salt mixture were prepared as previously described ('40). Yeast vitamins was a commercial product prepared from brewers' yeast. The label indicated that this product contained 240 B<sub>1</sub> and 60 B<sub>2</sub> Sherman units per gram. U.S.P. dextrose was used. Each diet was made up in the quantity of 1000 gm. at one

time and was stored in covered glass jars at 6°C. Diet no. 1 was practically free from fat and was fed to rats in order to determine the excretion of metabolic fat. In the diets containing 5, 15, 30, and 54.2% of fat, respectively, fat furnished approximately 12.5, 32.7, 54.5, and 77.7% of the total energy value. In the diets containing the different proportions of fat, the proportions of casein, yeast vitamins, and salts were adjusted so as to have approximately constant relationship to the total energy value.

#### EXPERIMENTAL PROCEDURE

The procedure in these experiments was similar to that previously followed by the writers ('40) in a study of the nutritive properties of certain animal and vegetable fats. Since the methods are described in detail in that publication, only a brief description will be given here.

The melting point of the fats was determined by the capillary tube method, the iodine number by the Hanus method, and the thiocyanogen number by the method of Kaufmann ('26). An approximately 0.1 N solution of thiocyanogen in redistilled glacial acetic acid was used. An excess of 100 to 150% of the freshly prepared reagent was used and absorption was conducted for 24 hours in the dark at room temperature (about 25°C.). Analyses were made in duplicate.

The percentages of linoleic, oleic, saturated, and unsaturated fatty acids were calculated from the iodine and thiocyanogen numbers by the formula given by Kass, Lundberg and Burr (p. 53, '40). In using this formula, the presence in lard of such a small proportion of arachidonic acid as has been reported by Ellis and Isbell ('26), and Brown and Deck ('30), would only slightly affect the calculated percentage of linoleic acid.

Before finally deciding upon the use of the formula proposed by Kass et al. ('40) in preference to that given by Jamieson ('32), some comparisons were made which showed a difference of approximately 0.4 in actual values for linoleic acid in the samples used in the present study. It is worthy

of mention that Kass et al. ('40) found elaidic acid to have the same thiocyanogen value as normal oleic acid.

In the growth experiments, all of which were conducted at the same time, each diet containing 5, 15, 30, or 54.2% of fat was fed to eight male albino rats for 60 days. The rats weighed approximately 40 gm. each at the beginning of the test and animals from different litters were distributed evenly among the different groups. Each rat was kept in an individual cage and an accurate record of food consumption obtained.

A digestion test was conducted for 7 days with each rat after the growth experiment had been in progress about 50 days. The feed consumed during the 7-day period was weighed and all feces excreted during the same period were collected. The feces were dried to constant weight at 100°C. and were analyzed for fat by the method previously described by the writers ('40). In these experiments, digestion and saponification of the feces were carried on in a 200 cc. Erlenmeyer flask with the addition of 25 cc. of 30% potassium hydroxide solution and 50 cc. of 95% alcohol.

At the end of the growth experiments, each rat was fed a fat-free diet and, after a preliminary period of at least 2 days, the feces were collected for 7 days. The feces were dried to constant weight at 100°C. and were analyzed for fat by the method previously described. The percentage of fat in the feces of a rat while on the fat-free diet was considered to be metabolic fat, and this figure was used in correcting for the metabolic fat in the feces of the same rat in the previous digestion test.

The true digestibility of each fat was calculated in the usual manner after making correction for the quantity of metabolic fat excreted during the digestion test. It was assumed that each rat excreted the same quantity of metabolic fat, in proportion to nonfatty dry matter, as when fed the fat-free diet.

STATISTICAL PROCEDURE<sup>1</sup>

In the analysis of the data, variance and covariance analyses as described by Snedecor ('37) were used. Adjustment of the gains to 3000 cal. was made using the within group regression coefficient between calories consumed and gain in weight. The adjustment was made to 3000 cal. merely for ease of calculation, yet this figure is within the range of the population. The minimum significant differences were obtained by multiplying the standard deviation of the mean differences calculated from the within group mean squares by the "t" values for odds of 15-1 and 99-1.

TABLE 2  
*Composition of fats used in feeding tests with rats*

TYPE OF SHORTENING	MELT- ING POINT	IODINE NUMBER	THIOCY- ANOGEN NUMBER	FATTY ACIDS AS PERCENTAGES OF TOTAL FATTY ACIDS <sup>1</sup>			
				Saturated fatty acids	Unsatu- rated fatty acids	Oleic acid <sup>2</sup>	Linoleic acid <sup>3</sup>
Steam-rendered lard	43	66.6	54.8	39.5	60.5	47.1	13.4
Hydrogenated cottonseed oil	39	70.8	59.2	34.5	65.5	52.3	13.2

<sup>1</sup> Calculated by formula given by Kass, Lundberg and Burr (pp. 53, '40).

<sup>2</sup> Includes isoöleic acid.

<sup>3</sup> May include arachidonic acid.

## EXPERIMENTAL RESULTS

The composition of the fats used in the experiments is shown in table 2. It will be seen that the lard had a higher melting point and contained a higher percentage of saturated fatty acids than the hydrogenated cottonseed oil. The two products contained approximately the same proportion of linoleic acid. This hydrogenated cottonseed oil contained a considerably higher proportion of saturated fatty acids than was previously found by the writers ('40) in another brand of hydrogenated cottonseed oil. This was undoubtedly due,

<sup>1</sup> Acknowledgment is made to Bradford Knapp, Jr., of the Animal Husbandry Division of the Bureau of Animal Industry, United States Department of Agriculture for the statistical analysis of the experimental data.

in part at least, to the addition of fully hydrogenated cottonseed oil to the partially hydrogenated product used in the present experiments.

#### GROWTH-PROMOTING VALUES OF FATS

Only the average growth-promoting values of steam-rendered lard and hydrogenated cottonseed oil are shown in table 3, but the data for individual rats have been analyzed by statistical methods and pertinent results are included in the

TABLE 3

*Average growth-promoting properties of steam-rendered lard and hydrogenated cottonseed oil in tests with male albino rats*

TYPE OF SHORTENING	FAT IN DIET	FEED CON- SUMED	GAIN IN WEIGHT IN 60 DAYS		
			Unad- justed	Adjusted to 3000 cal. intake	Per 100 cal.
Steam-rendered lard	%	<i>Cal.</i>	gm.	gm.	gm.
	5	3151	243	229	7.68
Hydrogenated cottonseed oil	5	2941	231	226	7.47
Difference		210	21	3	0.21
Steam-rendered lard	15	3149	267	253	8.48
Hydrogenated cottonseed oil	15	3279	260	234	7.92
Difference		130	7	19	0.56
Steam-rendered lard	30	3638	311	252	8.53
Hydrogenated cottonseed oil	30	3411	268	230	7.84
Difference		227	43	22	0.69
Steam-rendered lard	54.2	3398	297	260	8.73
Hydrogenated cottonseed oil	54.2	3193	251	233	7.86
Difference		205	46	27	0.87
Minimum significant difference (odds 19-1)		296	29	10	0.35
Minimum significant difference (odds 99-1)		394	39	14	0.46
Steam-rendered lard, average all levels		3234	280	249	8.36
Hydrogenated cottonseed oil, average all levels		3206	250	231	7.77
Difference		28	30	18	0.59
Minimum significant difference (odds 19-1)		148	15	5	0.17
Minimum significant difference (odds 99-1)		197	19	7	0.23

table. Each of the sixty-four rats used in these tests was in excellent condition at the end of the 60-day experiment, indicating that each fat was perfectly wholesome even when it constituted 54.2% by weight or 77.7% of the total energy value of the diet.

Feed consumption, expressed in terms of calories, did not differ significantly at any level of fat intake between rats fed lard and those fed hydrogenated cottonseed oil. The average feed consumption for all the rats fed diets containing lard differed only slightly from the feed consumption of all the rats fed diets containing hydrogenated cottonseed oil.

A comparison between the feed consumption of rats fed diets containing different proportions of each fat shows that in each case the feed intake was greatest when the diet contained 30% of fat. Feed consumption was least with cottonseed oil when the diet contained 5% of fat, and with lard feed consumption was lowest when the diets contained 5 and 15% of fat. The differences in feed consumption were highly significant for each fat.

The average unadjusted gains made by the rats show that, with each fat, the greatest gain was made when the diet contained 30%, and the smallest gain when the diet contained 5% of fat. The average unadjusted gain for all rats fed lard was significantly greater than the average gain for all rats fed hydrogenated cottonseed oil.

When the feed intake of all the rats was adjusted to 3000 cal., the adjusted gains show that lard and hydrogenated cottonseed oil had approximately the same growth-promoting value when the diets contained 5% of fat, but lard was definitely superior when the diets contained 15, 30, and 54.2% of fat. When the results were compared on the basis of gain in weight per 100 cal. consumed, the relative growth-promoting values of the two fats were as indicated above.

#### DIGESTIBILITY OF FATS

The average results of the digestion experiments with steam-rendered lard and with hydrogenated cottonseed oil

are shown in table 4. The data for each fat at each level of intake represent the average of the results obtained with eight rats. A statistical analysis of the data for individual rats shows the minimum difference necessary to be significant.

TABLE 4

*Average digestibility of steam-rendered lard and hydrogenated cottonseed oil in 7-day tests with male albino rats*

TYPE OF SHORTENING	FAT IN DIET	CRUDE FAT IN FECES		True digesti- bility
		On fat diet	On fat-free diet	
Steam-rendered lard	5	13.61	6.48	96.18
Hydrogenated cottonseed oil	5	21.17	6.47	92.30
Difference		7.56	0.01	3.88
Steam-rendered lard	15	27.52	7.03	95.89
Hydrogenated cottonseed oil	15	42.21	7.13	90.97
Difference		14.69	0.10	4.92
Steam-rendered lard	30	36.06	6.69	95.90
Hydrogenated cottonseed oil	30	48.63	6.86	93.17
Difference		12.57	0.17	2.73
Steam-rendered lard	54.2	34.47	6.62	97.44
Hydrogenated cottonseed oil	54.2	50.49	6.62	94.96
Difference		16.02	0.00	2.48
Minimum significant difference (odds 19-1)				1.50
Minimum significant difference (odds 99-1)				1.99
Steam-rendered lard, average all levels		27.92	6.71	96.35
Hydrogenated cottonseed oil, average all levels		40.63	6.77	92.85
Difference		12.71	0.06	3.50
Minimum significant difference (odds 19-1)				0.75
Minimum significant difference (odds 99-1)				1.00

The data show that steam-rendered lard was superior in digestibility to hydrogenated cottonseed oil at each level of intake, the difference being greatest when the diets contained 15%, and least when they contained 54.2% of fat. The average

digestive coefficient for lard at all levels of intake was materially greater than the average value for hydrogenated cottonseed oil. In each case the difference was highly significant.

The digestibility of lard was practically the same regardless of the proportion of fat in the diet. On the other hand, the digestive coefficient of hydrogenated cottonseed oil was significantly higher when the diet contained 54% than when it contained 5 or 15% of fat.

It is noteworthy that, at each level of fat intake, the feces from the rats fed hydrogenated cottonseed oil contained a considerably higher proportion of crude fat than the feces from the rats fed lard. On the other hand, after the rats had been changed to the fat-free diet, there was a marked uniformity in the proportion of crude fat in the feces.

#### DISCUSSION OF RESULTS

The results of the present experiments concerning the relative nutritive properties of steam-rendered lard and hydrogenated cottonseed oil confirm the results of previous experiments by the writers ('40). In each series of experiments the two fats had practically the same growth-promoting value at the 5% level of intake, but lard was superior at the higher levels. Lard was superior in digestibility at all levels of intake.

In the present experiments, the available data do not supply an adequate explanation for the differences found between the nutritive properties of the two fats. As regards the relative digestibility of the two fats, although lard had a slightly higher melting point and contained a considerably higher percentage of saturated fatty acids than hydrogenated cottonseed oil, lard had the higher digestive coefficients. The percentage of stearic acid was not determined, but on the basis of available data concerning the composition of lard and cottonseed oil (Dean, '38; Hilditch, '40), it seems probable

that the two fats contained approximately the same proportion of this fatty acid. The hydrogenated cottonseed oil, of course, contained a considerable proportion of isoöleic acid. Whatever the real explanation for the differences in the digestibility of steam-rendered lard and hydrogenated cottonseed oil may be, it appears clear that the changes induced in refined cottonseed oil in the process of hydrogenation lowered its digestibility. In previous experiments ('40) the writers found that refined cottonseed oil had as high a digestive coefficient as refined steam lard, whereas hydrogenated cottonseed oil had a considerably lower value.

An explanation for the superior growth-promoting properties of steam-rendered lard as compared with hydrogenated cottonseed oil is not apparent. The two fats contained approximately the same proportions of linoleic acid, so this fatty acid apparently was not a factor. Although the percentage of linoleic acid found in the hydrogenated cottonseed oil used in this experiment may seem rather high, yet it is similar to the results obtained by the writers with a number of other samples obtained from different sources. Spadola and Ellis ('36) found 12% of linoleic acid in a partially-hydrogenated cottonseed oil with an iodine number of 68.8. The possibility has been suggested that the percentage of linoleic acid in a partially-hydrogenated cottonseed oil, as calculated from the iodine and thiocyanogen numbers, might not be valid. The reason given was that the isoöleic acids in the hydrogenated fat might have different thiocyanogen numbers than normal oleic acid. This assumption seems unwarranted since Kass, Lundberg and Burr ('40) found elaidic acid to have practically the same thiocyanogen number as oleic acid.

Although arachidonic acid was not determined in the lard used in the present experiments, it seems unlikely that such a small proportion of this acid as has been previously reported in commercial lard would materially affect the growth-promoting value of a lard such as was used by the writers.

It is noteworthy that the rat can utilize a diet containing 54.2% fat and no carbohydrates as efficiently for growth as diets containing smaller proportions of fat together with dextrose. The rats on the high-fat diets were in excellent condition throughout the experiment.

The high digestibility of steam-rendered lard at all levels of intake indicates the unusual capacity of the rat for the absorption of this fat. Although the digestibility of hydrogenated cottonseed oil was significantly lower than that of lard at each level of intake, yet the vegetable fat was well utilized even when the diet contained the maximum proportion of fat.

#### SUMMARY

The relative growth-promoting properties and digestibility of steam-rendered lard and hydrogenated cottonseed oil were determined by experiments with young male albino rats. Each fat was incorporated in an otherwise adequate diet in the proportions of 5, 15, 30, and 54%.

The two fats had approximately the same growth-promoting value when the diets contained 5% of fat, but lard was superior when the diets contained the larger proportions of fat. The average growth-promoting value of all diets containing lard was superior to the average value of all diets containing hydrogenated cottonseed oil.

Both lard and hydrogenated cottonseed oil induced maximum growth when the diets contained 30%, and minimum growth when the diets contained 5% of fat. Each fat was utilized least efficiently for growth when the diet contained 5% of fat, but each was utilized with approximately equal efficiency whether the diet contained 15, 30, or 54% of fat.

Lard was superior in digestibility to hydrogenated cottonseed oil at each level of fat intake. The digestive coefficients for lard ranged from 95.9 to 97.4% with an average of 96.4% whereas those for hydrogenated cottonseed oil ranged from 91.0 to 95% with an average of 92.9%.

## LITERATURE CITED

- BROWN, J. B., AND E. M. DECK 1930 The occurrence of arachidonic acid in lard. *J. Amer. Chem. Soc.*, vol. 52, pp. 1135-1138.
- DEAN, H. K. 1938 Utilization of fats. 306 pp., illus. New York.
- ELLIS, N. R., AND H. S. ISBELL 1926 Soft pork studies II. The influence of the character of the ration upon the composition of the body fat of hogs. *J. Biol. Chem.*, vol. 69, pp. 219-238.
- HILDITCH, T. P. 1940 The chemical constitution of natural fats. 438 pp., illus. London.
- HOAGLAND, RALPH, AND GEORGE G. SNIDER 1940 Nutritive properties of certain animal and vegetable fats. *Tech. Bull.* 725, 12 pp. U. S. Dept. of Agriculture.
- JAMIESON, GEO. S. 1932 Vegetable fats and oils; the chemistry, production and utilization of vegetable fats and oils for edible, medicinal and technical purposes. 444 pp., illus. New York.
- KASS, J. P., W. O. LUNDBERG AND G. A. BURR 1940 The linoleic acid content of seed fats and isomerism of linoleic acid. *Oil and Soap*, vol. 17, pp. 50-53.
- KAUFMANN, H. P. 1926 Die Rhodanometrie von Fetten und Fettgemischen. *Ztschr. f. Untersuch der Nahr. u. Genussmtl*, vol. 51, pp. 15-27.
- SNEDECOR, GEORGE W. 1937 Statistical Methods, 341 pp., illus. Ames.
- SPADOLA, JOHN M., AND N. R. ELLIS 1936 The effect of the ingestion of cotton-seed oil before and after hydrogenation on the composition of the body fat of the rat. *J. Biol. Chem.*, vol. 113, pp. 205-218.

### III. AVIAN THIAMINE DEFICIENCY CHARACTERISTIC SYMPTOMS AND THEIR PATHOGENESIS<sup>1</sup>

ROY LAVER SWANK AND OTTO A. BESSEY

*Department of Pathology, Harvard Medical School, The Harvard Dental School,  
and the Medical Clinic of the Peter Bent Brigham Hospital,  
Boston, Massachusetts*

ONE FIGURE

(Received for publication December 17, 1940)

Thiamine deficient pigeons usually exhibit opisthotonus (head retraction) and, in fewer instances, leg weakness and hydropericardium (McCarrison, '19; Findlay, '21). The latter two symptoms have received little attention in recent years, and some investigators (Reader, '30; Kline et al., '36) have considered leg weakness (in the rat and chick) to be due to another deficiency, namely vitamin B<sub>1</sub>. By others (Prickett et al., '39), however, it has been described in the rat as a manifestation of chronic vitamin B<sub>1</sub> deficiency. In humans, leg weakness and cardiac failure usually develop as a result of vitamin B<sub>1</sub> deficiency, whereas opisthotonus must occur rarely if at all. Spasticity (which may be related to opisthotonus) has been observed in infants.

This apparent lack of uniformity within a clinical entity is not unusual and would appear superficially to be explained adequately by individual and species differences. However, most experimental thiamine deficient diets are practically free from this factor which is a degree of restriction of thiamine in the diet rarely if ever attained in a human's ration. Moreover, various other factors, i.e., starvation, alter the severity

<sup>1</sup>This investigation was aided by a grant from the William W. Wellington Memorial Research Fund of Harvard University.

and course of a developing deficient state, and might possibly affect the individual variations observed so commonly in the pigeon, and be an important factor in determining the symptomatology in human beriberi.

In the present study the thiamine and food intake were varied to produce a deficiency and starvation of varying severity, and the symptomatology studied in an attempt to find a clue to the pathogenesis of the different manifestations of thiamine deficiency in the pigeon.

#### MATERIALS AND METHODS

Unless otherwise stated all of the observations reported in this paper were made upon white Karneaux pigeons that were 6 to 8 weeks old at the beginning of experimentation. They were fed one of three vitamin B<sub>1</sub> free diets. Diet I was consumed ad libitum and consisted of mixed grain (Canadian peas, Kaffir corn, yellow corn, and hard wheat) which had been autoclaved for 6 hours at 20 pounds of pressure. The behavior and food intake of birds that consumed this diet were so variable that synthetic diets II and III, which could be fed by tube, were devised. Diet II contained casein (alcohol-extracted) 20%, corn starch 65%, cod liver oil 3%, peanut oil 8%, salt mixture 4%, and vitamin K concentrate.<sup>2</sup> This diet was practically free of both vitamin B<sub>1</sub> (thiamine) and the known water-soluble members of the B complex group of vitamins. Thus in thiamine curative experiments any rehabilitation which occurred could be attributed to none other of the B group of vitamins than thiamine. Diet III contained the ingredients of diet II plus 15% autoclaved yeast, and was utilized as a control for diet II. The proportions of casein, starch, and peanut oil in diet III were changed so that the protein, carbohydrate, and fat contents of this diet were the same as in diet II.

Diet I was consumed voluntarily, but diets II and III were administered by tube as follows. The birds were secured in a

<sup>2</sup> Abbott.

holder;<sup>3</sup> a soft rubber tube  $\frac{1}{4}$  inch diameter  $\times$  5 inch length was inserted into the crop; and a weighed amount of diet, previously made into a paste by the addition of an equal amount of water, was forced through the tube from a catheter syringe. This method of feeding diets II and III was employed with no harmful results in more than 300 pigeons, and some birds were fed diet III in this manner for as long as 6 and 8 months. Thiamine supplements<sup>4</sup> were dissolved in distilled water and injected intramuscularly.

Subsequent to preliminary experimentation with both diets II and III, the food (diet II) and thiamine intake were varied in sixty pigeons (fig. 1). Twenty-four of these were each fed 20 gm., and the remaining thirty-six either 10 or 5 gm. of diet II daily. After two birds in each group vomited, all birds in that group were given a daily supplement of thiamine intramuscularly, ranging from 0 to 25  $\mu$ g. for birds on the 20-gm. intake, 0 to 12  $\mu$ g. for those on 10-gm. intake, 0 to 7  $\mu$ g. for those on the 5-gm. intake. These birds were carefully observed daily for signs of opisthotonus, ataxia, leg weakness, decreased activity, regurgitation and dyspnea, and when these symptoms had been present for a desired period of time, 50 to 200  $\mu$ g. of thiamine were given intramuscularly each day until partial or complete rehabilitation had occurred. During the period of repair most birds continued to receive diet II, but some of them, for the sake of convenience, were fed normal grain.

Many other pigeons were used for studies relative to the production of hydropericardium and other findings compatible

<sup>3</sup> The holder is made of  $\frac{1}{4}$  inch wood stock on a base 12 inches  $\times$  6 inches. Four small pieces of wood are mounted edgewise and at angles to fit the contour of the pigeon's body when on its back with wings closed. One side of two pieces at 150 degree angle is fixed to the base. The other two pieces can be moved in slots and about the axis of bolts, used to secure their position, in a manner to obtain a snug fit. One slot is parallel and the other at a 45 degree angle to the edge of the base. An oblong enclosure of varying dimensions is thus obtained. The pigeon's head protrudes through a hole formed by matched notches in the two end pieces when they are in position.

<sup>4</sup> Betabione, Merck.

P	G	B,	Y	D	5	10	15	20	25	30	35	40
65	20	—	—	—	V	0						
66	20	—	—	—	V	0						
67	20	—	—	—	V	0						
68	20	5	25	6		A	0					
69	20	5	25	6			A0					
70	20	5	25	6			0					
142	20	10.5	9		V	A		X				
141	20	15	75	10	V	A		X				
140	20	15	75	6	V				A	X		
138	20	15	75	7	V		A	X				
137	20	20	1.0	10	V	A		X				
136	20	20	1.0	9	V			Continue to vomit				
134	20	20	1.0	7	V			Continue to vomit				
133	20	25	25	6	V						X	
132	20	25	25	8	V						X	

152	10	—	—	—			0					
155	10	7	7	12		V	A		X			
154	10	7	.7	13		V	A		X			
153	10	7	.7	11		V		A		X		
151	10	10	0	11		V					A	X
150	10	10	0	10	V					A		X
149	10	10	0	9	V					A		X
148	10	10	0	14		V	A	0				
147	10	12	1.2	10	V						X	
146	10	12	1.2	11	V						X	

157	5	—	—	—			A	0				
158	5	—	—	—			A	0				
167	5	—	—	—				A0				
166	5	3	.6	16				A		X		
165	5	3	.6	14		V		A	X			
164	5	3	6	16			A		X			
163	5	5	1.0	14		V	A		X			
162	5	5	1.0	14		V						X
161	5	5	1.0	16			A	X				
156	5	7	1.4	14							X	
159	5	7	1.4	14		V					X	

Figure 1

with cardiac failure. The details of these experiments will be described in the text.

#### RESULTS

*Diet I.* The behavior of thirty or more birds that consumed diet I (heated mixed grain) voluntarily was essentially the same as was noted by McCarrison ('19) and Findlay ('21). Ataxia appeared on about the nineteenth day and was followed in 1 to 13 days by opisthotonus. Anorexia was usually noticed by the seventh to tenth day, and nearly every bird lost 20 to 35% of its original weight by the time that neurological symptoms had appeared. One pigeon that was given inadequate thiamine supplements for 11 weeks and then deprived of thiamine entirely developed cardiac failure with pulmonary edema and hydropericardium.

*Diet II* (fed by tube). It was known from preliminary experimentation that a pigeon could maintain or gain weight and remain active and in good health for 8 to 12 weeks, when fed by tube a daily ration of 20 gm. of diet II plus an intramuscular injection of 25 µg. or more of thiamine. Later, anorexia, a loss of weight and anemia appeared, and were not influenced by increasing the thiamine intake. Consequently no experiments with diet II exceeded 8 weeks, and, unless specifically stated, none exceeded 6 weeks in duration.

Reference to figure 1 shows that vomiting<sup>5</sup> (anorexia) occurred on or about the eighth experimental day in almost all birds receiving 20 gm. of diet daily and no thiamine. This

<sup>5</sup> The food was spilled apparently by lowering and shaking the head.

Fig. 1 The clinical observations upon birds that were fed 20, 10, and 5 gm. of diet II daily are included in the figure. In the first vertical column (P) is to be found the number of all pigeons referred to by number in the paper; in the second column (G) is noted the daily ration (in grams of diet II); in the third ( $B_1$ ) can be found the daily thiamine intake (in micrograms); in the fourth ( $\gamma/g$ ) the ratio of micrograms of thiamine/grams of diet II; and in the fifth (d) the day on which thiamine was started. The horizontal row of numbers from 5 to 40 at the top of the chart gives the experimental day. The letters in the chart have the following meaning: V—Vomiting; O—Opisthotonus (immediately repaired or sacrificed); A—Ataxia; X—Experiment discontinued; pigeon given reparative doses of thiamine or sacrificed.

was both delayed and reduced in quantity by reducing the daily ration to 10 gm., and absent or greatly delayed in birds that received 5 gm. of diet II daily.

When thiamine was added the vomiting was decreased roughly proportional to the size of the dose so that many of the chronically deficient pigeons lost no food by this means.

Due to this plan of feeding and the behavior of the birds under these conditions, the average loss of weight at the time neurological signs first appeared was only 5% in those receiving 20 gm.; 15% in those receiving 10 gm. and 24% in those receiving 5 gm. of food, in marked contrast to the large uncontrollable losses in weight which usually occur with voluntary feeding.

#### *Neurological manifestations*

The clinical manifestations of thiamine deficiency in these experiments varied (fig. 1) depending upon the rate of onset of the deficiency and this rate was dependent upon the ratio of thiamine intake to food intake. The acute deficiency produced by feeding the thiamine-free diet in amounts sufficient to prevent a marked weight loss always led to opisthotonus except in very old birds (P. 65, 66, 67).<sup>6</sup> The more acutely the deficiency developed, the greater the tendency seemed to be for opisthotonus to be severe and sustained, accompanied by backward rolling and extensor rigidity of the legs, and flapping of the wings. When the deficiency developed less rapidly because of concomitant starvation (157, 158 and 167) or the addition of small amounts of thiamine to the diet (68, 69, and 148) ataxia preceded the opisthotonus. If the thiamine supplements were greater and the deficiency in thiamine developed slowly, ataxia and later leg weakness appeared (137, 138, 140, 141, and 142).

The earliest evidence of leg involvement was ataxia, which appeared as early as the thirteenth day, but usually later.

<sup>6</sup> All pigeons referred to by number are to be found in the chart.

It was characterized by a wide gait, misplacing of the feet, anterior buckling of the knees, and staggering and was frequently followed by definite muscular weakness so that the birds were unable to support their weight. The paralysis developed rapidly when the thiamine intake was low, and in 1 to 3 days the birds were unable to stand, although they could still flap their wings and support their heads with no obvious difficulty. Many birds were maintained in this state for 7 to 14 days, and one for 4 weeks, after which they remained paralyzed for a variable period during repair. A few birds with a nearly adequate thiamine intake exhibited definite mild ataxia 1 day and normal gait the next, for as long as 3 weeks before the paralysis became definitely sustained, and marked leg weakness, without buckling of the knees and misplacing of the feet, could be produced temporarily in others by exercise.

All cases of leg weakness or opisthotonus that appeared in animals on diets I, II, and III were alleviated by therapeutically adequate intramuscular injections of thiamine. Opisthotonus of short duration was relieved in approximately 1 or 2 hours by 50 to 100 µg. of thiamine, but smaller doses of thiamine required as long as 8 to 12 hours. A few birds on the starvation and deficient diets (and a few receiving autoclaved grain) recovered spontaneously from opisthotonus for a day or so, but in none of the well-fed acutely deficient birds was this observed. This was thought to be due to the ability of these animals to give up their tissue stores of thiamine, or to various other factors which conserve the animal's supply of vitamin B<sub>1</sub>, i.e., fasting, vomiting, and coprophagy. The symptoms of ataxia or leg weakness were abolished much more slowly. Birds with only ataxia for a day or less were entirely free of symptoms in 1 or 2 days on a dosage of 50 to 100 µg. of thiamine, but leg weakness (paralysis) severe enough to make standing difficult for 3 days, delayed complete clinical rehabilitation for 2 to 4 weeks (three experiments), and if the paralysis had existed for 7 to 9 days, restoration to normal was delayed for 6 to 8 weeks (six

experiments). A number of birds with moderate leg weakness improved spontaneously for a few days while being maintained on thiamine intake slightly inadequate for their daily need, although no change in the food or thiamine intake was made. Later the paralysis reappeared and was abolished only after definite curative doses of thiamine were given. Although barely adequate doses of thiamine were curative, it seemed wise in these experiments to give what was found later to be a large excess (50 to 100  $\mu\text{g}$ . daily).

Recovery from leg weakness was successful in nine birds that received diet II plus 50 to 100  $\mu\text{g}$ . of thiamine daily. Two of these with severe symptoms improved slowly while receiving the starvation ration of 5 gm. daily, but much more rapidly after the food intake was doubled, although the thiamine intake was unchanged and the caloric intake still insufficient for normal growth. Two other birds that were recovering satisfactorily on diet II plus thiamine, developed other deficiencies after the experiment had exceeded the 8 to 12 week limit and exhibited anemia and weight loss, but an increase in the paralysis was not noted.

The data in figure 1 confirm the previously reported (Cowgill, '39) dependence of thiamine requirements on food intake; 1 gm. of diet II requires about 1.25  $\mu\text{g}$ . of thiamine to prevent all symptoms. Thus it is not surprising that birds which continue to vomit (134, 136) failed to develop paralysis; other birds (140, 138, 151, 150, 149) that vomited early, developed ataxia late; and those that vomited late (141, 148) developed ataxia early. The vitamin economy affected by vomiting is also illustrated by older birds that received 20 gm. of diet II daily and failed to vomit. They occasionally developed opisthotonus in 9 to 11 days, whereas the younger birds that vomited required 13 to 15 days and occasionally longer to develop this symptom.

Concomitant starvation tends to make a deficiency state appear more chronic (157, 158, 167) probably because a lowered food intake allows the rapidly depleting tissue stores

to be used at a slower rate, so that these birds resemble those on higher food intake which receive small supplements of thiamine (68, 69, 148).

*Cardiac failure: its production and alleviation*

Evidences of cardiac failure (pericardial effusion, dyspnea, engorgement and edema of lungs, engorgement of the liver and (or) dependent edema of the body) were observed rarely in the acutely deficient pigeons (such as 65, 66, 67) with opisthotonus only, and never in the severely starved and deficient ones. However, in a group of eleven chronically deficient birds with leg weakness that received a daily ration of approximately 15 gm. of food and 5 to 10  $\mu$ g. of thiamine for 25 to 32 days (similar to 68, 69, and 70), nine had one or more postmortem evidences of incidental cardiac failure, and two that had been given large doses of thiamine for purposes of repair were found normal at autopsy 4 days and 1 month later. Of those cases with evidence of cardiac failure, pericardial effusion, apparently alone, was found in three cases, with pulmonary edema in four, and pulmonary engorgement of the liver and edema of the pectoral regions and thighs in one. In no one of these eleven chronically deficient pigeons was dyspnea or other antemortem evidence of cardiac failure observed.

In ten other chronic cases on a similar chronic dietary regimen, severe acute cardiac failure indicated clinically by dyspnea was produced by suddenly discontinuing the thiamine. These birds had received a daily ration of 20 gm. of diet II, and, subsequent to vomiting, 10 to 20  $\mu$ g. of thiamine for 1 to 4 weeks. The thiamine was then withdrawn, and 1 to 4 days later dyspnea developed. Despite thiamine medication five of these birds died less than 1 hour after dyspnea was noted and their postmortem studies revealed very marked engorgement and edema of the lungs, engorgement of the liver and pericardial effusion. Occasionally this severe type of cardiac failure with dyspnea was seen in birds that were maintained on a prolonged chronic deficiency regime with a

continued intake of inadequate doses of thiamine, and a few of these birds developed opisthotonus terminally. It should be noted that some pigeons with either incidental or severe cardiac failure had slight to moderate leg weakness, but opisthotonus was observed rarely except as a terminal event.

Incidental cardiac failure was readily eliminated by injections of thiamine as was indicated by the absence of these pathological findings in all the successfully treated birds included in the chart. Furthermore, in a group of twelve chronically deficient birds, all of which were treated in an identical fashion, hydropericardium and other evidences of incidental cardiac failure were observed at postmortem in six birds, and one appeared normal. The five remaining birds were given 100 µg. of thiamine daily and autopsied after 1, 2, 3, 4, and 5 days. No evidence of cardiac failure could be found. On the other hand, five pigeons with severe cardiac failure and dyspnea failed to respond to thiamine and died within a few minutes of its administration. Five others, however, responded to this medication and were apparently normal in 30 to 60 minutes.

*Diet III* (fed by tube). The foregoing experimentations with diet II were repeated with diet III on fewer birds. The clinical observations were essentially the same in the two groups, so this group will not be described separately.

#### DISCUSSION

It appears that pigeons allowed to feed voluntarily on thiamine deficient diets will unpredictably become acutely or chronically deficient, depending upon the intensity of their appetites and the amount of thiamine which they consume. A few birds will eat the deficient diet voraciously and develop opisthotonus in 14 days while others, because of starvation, will develop a chronic deficient state or die from anorexia. This is the principal reason for the general failure to consistently produce satisfactory opisthotonus in pigeons for assay purposes.

If the diet consists of poorly-milled rice or mixed grain insufficiently autoclaved, or if wire-bottomed cages are not used or are cleaned poorly so that fecal matter is available, an unknown and occasionally adequate amount of thiamine will be obtained. Under such circumstances, pigeons will develop a chronic deficiency (no opisthotonus) or none at all.

It is important to recognize that acute and chronic thiamine deficiency, although different in many respects, represent two phases of the same deficiency. It seems probable that many of the inconsistencies in the literature concerning vitamin B<sub>1</sub> deficiency are due to a failure to recognize that symptoms of a deficiency may vary depending upon the manner in which the deficiency is produced. This ought to be kept in mind, especially when proposing a new factor as an explanation for inconsistent symptoms and lesions. The vitamin B<sub>4</sub> hypothesis seems to be a case illustrating this point.

The immediate response of the acutely deficient birds with opisthotonus to thiamine treatment indicates that faulty function is primarily responsible for this symptom. The alleviation of mild ataxia and some prompt but limited improvement in leg weakness indicate that a functional factor is also probably involved in the chronic deficiency. However, the fact that complete rehabilitation of leg weakness requires from several days to several weeks, depending upon the time at which repair is instituted, is almost conclusive evidence that anatomical changes are also involved in these cases. These observations have a bearing on the controversial point as to whether peripheral nerve degeneration is involved in thiamine deficiency. The above evidence plus the detailed pathological findings reported elsewhere (Swank, '40) show that leg weakness is accompanied by peripheral nerve degeneration, the extent of which depends on the time factor; and that upon repair with thiamine on a diet entirely devoid of other factors of the B complex, the peripheral nerves regenerate parallel with clinical improvement in the symptoms. It seems clear that experimental thiamine deficiency can exist with symp-

toms due either to a functional or an anatomical lesion, or both, depending upon the manner in which it is produced.

One cannot dismiss the possibilities that species differences may also influence the type of symptoms which will develop. Perhaps opisthotonus is produced easily in the pigeon because of the highly developed vestibular system. In ducks, opisthotonus was also produced with ease, but in chickens with greater difficulty, although identical dietary methods were used. The age is also an important factor; leg weakness and opisthotonus were most easily produced in young growing or adult birds, while old birds have a marked tendency to develop less conspicuous symptoms.

These experiments suggest that man usually develops the chronic type of thiamine deficiency (muscular tenderness, leg weakness and cardiac failure or both). This is consistent with the fact that his diet is usually not entirely free of thiamine. Dogs also develop the chronic type of thiamine deficiency as they conserve their thiamine by voluntary starvation (or vomiting when tube-fed). In a separate study of thiamine deficiency in dogs (Swank et al., '41) in which tube-feeding was used, one dog that did not regurgitate developed extensor rigidity with opisthotonus. This was abolished within a short time by an intramuscular injection of thiamine. This observation and the fact that spasticity occurs in infants with beriberi suggest that opisthotonus might develop in man if a diet entirely free of thiamine were consumed in an amount sufficient to prevent a significant loss of weight.

In spite of possible species differences it would seem worthwhile to consider some of the observations reported here as contributing toward a better understanding of the human deficiency.

#### SUMMARY AND CONCLUSIONS

Young pigeons fed a highly purified thiamine-free diet by tube in quantities sufficient to prevent large weight loss invariably developed an acute thiamine deficiency characterized by opisthotonus. When the ration was made only partially

adequate by additions of thiamine, ataxia and leg weakness developed in all pigeons and cardiac failure in many.

The intramuscular administration of thiamine promptly relieved the opisthotonus and the mild cardiac failure. Leg weakness, however, was restored to normal only slowly, irrespective of the presence or absence of other factors of the "B complex" and severe cardiac failure often did not respond to treatment.

The thiamine requirement varied with the food intake, 1.25 µg. per gram of diet II were required for pigeons to remain symptom-free.

The assumption that the absence of a hypothetical factor (vitamin B<sub>4</sub>) is responsible for the development of a paralysis when the thiamine intake is low is unnecessary in the case of pigeons. This paralysis is a characteristic symptom of chronic thiamine deficiency. The question is raised as to whether this is not also the case for the chick and rat.

#### LITERATURE CITED

- COWGILL, G. R. 1939 The Physiology of Vitamin B<sub>1</sub>. The Vitamins. Symposium, J.A.M.A.
- FINDLAY, G. M. 1921 An experimental study of avian beriberi. J. Path. and Baet., vol. 24, p. 175.
- KLINE, O. L., H. R. BIRD, C. A. ELVEHJEM AND E. B. HART 1936 An improved synthetic ration for vitamin B<sub>4</sub> studies. J. Nutrition, vol. 11, p. 515.
- MCCARRISON, R. 1919 The pathogenesis of deficiency disease. Ind. J. M. Res., vol. 6, p. 275.
- PRICKETT, C. O., W. D. SALMON AND G. A. SCHRADER 1939 Histopathology of the peripheral nerves in acute and chronic vitamin B<sub>1</sub> deficiency in the rat. Am. J. Path., vol. 15, p. 251.
- READER, VERA 1930 The assay of vitamin B<sub>4</sub>. Biochem. J., vol. 24, p. 1827.
- SWANK, R. L. 1940 Avian thiamin deficiency. A correlation of the pathology and clinical behavior. J. Exp. Med., vol. 71, p. 683.
- SWANK, R. L., R. PORTER AND A. YEOMANS 1941 The production and study of cardiac failure in thiamin deficient dogs. Am. Heart J., in press.



# THE EFFECT OF DIETARY CALCIUM AND PHOSPHORUS ON THE ASSIMILATION OF DIETARY FLUORINE<sup>1, 2</sup>

MARGARET LAWRENZ AND H. H. MITCHELL

*Division of Animal Nutrition, University of Illinois, Urbana*

(Received for publication January 3, 1941)

There is a definite fluorine hazard in the United States and throughout the world. The occurrence of fluorine in drinking water is general and in many communities, especially those served by deep wells, the concentration is such (0.9 p.p.m. or more) as to cause mottled enamel in children during the period of tooth formation (Nichols, '39). McClure ('39) has summarized published analyses of the distribution of fluorine in foods. Excluding excessive values obtained in certain fluorite areas and values obtained for vegetables and fruits sprayed with fluorine-containing insecticides, some 42% of the analyses range from 0 to 0.9 p.p.m. on the dry basis, 35% range from 1 to 2.8 p.p.m. (the present Federal tolerance for fluorine for marketable sprayed produce), and 23% range above 2.8 p.p.m. In the latter classification are sea foods, teas, baking powders and some milling by-products and leguminous seeds. According to this summary, foods grown in fluorite areas may show greatly increased contents of fluorine. The considerable use of fluorine-bearing insecticides in the fruit industry (Carter and Busbey, '39), especially

<sup>1</sup> This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America and the Pennsylvania Salt Manufacturing Company.

<sup>2</sup> This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

in the northwest, accounts for another item in the usual fluorine hazard to which the population of this country is exposed. A further item, of no general prevalence it is true, is the exposure to fluorine in industry (Machle and Evans, '40).

Some of these items in the usual fluorine hazard are beyond control, while some are subject to control within limits, depending upon economic factors, so that the fluorine hazard cannot be removed or reduced below certain limits. It is important, therefore, to consider the possibility of alleviating the physiological effects of fluorine on the body by dietary means, in order intelligently to be able to minimize the deleterious effects of fluorine consumed in food and water in amounts that it is either impossible or inexpedient to reduce.

The possibility of modifying the physiological effects of fluorine by dietary means has been explored with varying results. In an earlier paper from this laboratory (Lawrenz, Mitchell and Ruth, '39 b) it was shown that fluorine in food is retained in the body to a less extent (about 20% less) than fluorine in water, when the water is consumed at times such that admixture with food in the stomach cannot occur. The failure of McClure ('39) to confirm this finding may be ascribed to the fact that in his experiments the consumption of water was not regulated, and since dry food was offered the rats, a preponderant share of the day's water intake was consumed simultaneously with the food.

In connection with such studies, it seems fair to presume that the effect of any dietary factor upon the toxicity of fluorine reflects accurately its effect on the retention of fluorine in the body, since no mechanism is known, or seems at all probable, by which fluorine could be retained in some innocuous form. Hence, the degree of retention of fluorine under a given set of experimental conditions may be taken to measure the intensity of the physiological effects of dietary fluorine. It possesses the additional advantage of being amenable to quantitative and exact measurement.

Smith ('35-'36) has reported the results of a general study of the effects of dietary factors on fluorine toxicity as

measured by the time of appearance of tooth striations and of bleaching of the dental enamel in the rat and by the severity of these effects. Excessive supply of dietary essentials did not nullify these effects of fluorine, while suboptimal intakes of any known dietary essential, including calcium, were not observed to induce any increased susceptibility to dental fluorosis, although additive effects were evident if these dietary deficiencies in themselves were capable of impairing tooth formation. However, as De Eds ('33) has shown in his excellent review of chronic fluorine intoxication, there have been many indications that dietary calcium exerts a protective action and that calcium deficient diets accentuate the symptoms of fluorosis. In fact, before the etiology of mottled enamel was established, Pierle ('26) concluded that a low-calcium diet could produce mottled and stained teeth. Hauck, Steenbock and Parsons ('33) reported that the growth of rats was poorer on a food containing 0.15% NaF when the calcium content was low than when it was adequate, and that a supplement of vitamin D reduced the toxicity of the calcium-poor food, but not that of the calcium-rich. Schulz ('38) has recorded similar experiences, largely of a qualitative nature.

In a recent publication from this laboratory (Shields and Mitchell, in press) it was shown that dietary calcium and phosphorus are protective against lead retention in the sense that moderate as compared with low dietary concentrations of these elements depress greatly the retention in the body of dietary lead. Because of this relationship, which had been observed (though not very precisely defined) before the initiation of the work above referred to, and because of rather clear indications, not without some seemingly contradictory evidence however, that calcium is protective against fluorine retention, the experiments described in this report were undertaken. Their object was to define as accurately as possible any relationship that may exist between the concentrations of dietary calcium and phosphorus and the retention of dietary fluorine. An essential feature of the investigation was the control of all gross factors, including the intake of food, that would be

expected to modify the retention of fluorine. The dietary fluorine was purposely kept low so that the results could be more readily applied to the fluorine hazard existing in practical nutrition.

#### PLAN OF THE EXPERIMENTS

The experiments were carried out on growing albino rats. The first experiment was concerned with the effect on fluorine retention of variable concentrations of dietary calcium, the concentration of dietary phosphorus being kept constant. Twelve pairs of littermate rats of an initial average body weight of 120 gm., and eight pairs of younger littermates, averaging 44 gm. in weight were used in comparing the effects of a low-calcium and of a high-calcium diet. The low-calcium diet contained 47.5% of ground yellow corn, 46% of dried whole egg, 3% of dried yeast<sup>3</sup>, 2% of a fortified cod liver oil<sup>4</sup> and 1.5% of BaSO<sub>4</sub>. To this ration a synthetic cryolite was added in a concentration equivalent to 8 p.p.m. of fluorine. By actual analysis, the ration contained 0.23% of calcium and 9.4 p.p.m. of fluorine. The ratio of Ca to P was 0.44 to 1.

One rat in each of the twenty pairs of rats received this low-calcium ration, while the other rat received a high-calcium ration prepared according to the above formula except for the substitution of CaCO<sub>3</sub> for the BaSO<sub>4</sub>. The high-calcium ration contained by actual analysis 0.73% of calcium and 9.4 p.p.m. of fluorine. In this case, the Ca:P ratio was 1.40 to 1.

The paired rats received the same amounts of their respective diets and were fed to the limit of their appetites. The feeding period for each pair was terminated when each rat had consumed 1 kg. of food, with the exception of two pairs in the group of larger rats. One rat in each of these pairs developed abscesses on the neck, bearing no apparent relation to the experimental treatment, and the two pairs were sacrificed after consuming only 557 and 627 gm. of food, respectively,

<sup>3</sup> Northwestern Yeast Co., Chicago, Ill.

<sup>4</sup> Nopeo XX.

per rat. At the termination of the feeding experiment, all rats were sacrificed for analysis, and at the beginning of the experiment, sample rats were taken for analysis from each of the litters that furnished rats for the experiment. These two series of analyses permitted the estimation of fluorine retention during the course of experimental feeding.

In the second experiment, a similar comparison was made among three rations containing the same concentrations of calcium and fluorine, but three different levels of phosphorus. The basal (low phosphorus) ration contained 7% of egg albumin, 10% of casein, 8% of dried yeast, 10% of sucrose, 1.4% of a salt mixture free of calcium, phosphorus and fluorine, 6% of corn oil, 6% of lard, 2% of cod liver oil, 0.5% of wheat germ oil, 46.05% of starch, 3.05% of  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , and sufficient synthetic cryolite to provide 10 p.p.m. of added fluorine. The phosphorus contents of the other two rations were adjusted to the desired levels without disturbing the calcium content by substituting for the  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  appropriate mixtures of  $\text{CaHPO}_4$ ,  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , and  $\text{BaSO}_4$ . The three rations contained, respectively, 0.14, 0.54 and 0.71% of phosphorus and 0.71% of calcium, but instead of the anticipated 10 to 12 p.p.m. of fluorine, they were found to contain 32 p.p.m., attributable to a brand of commercial casein with a high content of fluorine.

Four trios of rats, averaging 48 gm. in initial weight, were used in the comparison of these three rations. The experiment was terminated with the consumption of 500 gm. of food per rat, and the fluorine storages were estimated in the usual way.

The third experiment was similar to the second in purpose and plan. In order to reduce the fluorine content to the desired level, egg albumin was substituted for casein in the experimental rations, which contained, respectively, 0.18, 0.51, and 0.63% of phosphorus, 0.74% of calcium and approximately 13 p.p.m. of fluorine. Eight trios of rats were used in these comparisons, which were terminated after the consumption of 600 gm. of food per rat.

The experimental rats were weighed weekly and after the sixth week of feeding, the teeth were examined weekly for the appearance of striations, using a jeweler's lens with a magnification of 4x. At the completion of the feeding period, the rats were killed with ether and the empty weights and body lengths taken. The carcasses were then autoclaved and the flesh, bones and teeth separated and analyzed for fluorine by methods previously described (Lawrenz, Mitchell and Ruth, '39 a). In the first experiment the dried bones were also analyzed for ash, calcium and phosphorus by approved methods.

#### DISCUSSION OF RESULTS

*The effect of low- and high-calcium levels on fluorine retention.* Striations began to appear on the teeth rather generally among the low-calcium rats during the eighth week, and among the high-calcium rats either simultaneously with their pair mates or shortly thereafter. The low-calcium rats gained at a somewhat slower rate than their pair mates, but the differences were not sufficiently distinct to be significant. After periods averaging 106 and 98 days for the groups of small and large rats, respectively, the rats were killed and analyzed. The average results of the various measurements and analyses made on the carcasses are summarized in table 1, with statistical analyses of their significance according to the method of Student ('25). The larger and smaller rats are grouped separately in this table.

The high-calcium diet produced a distinctly heavier weight of dry fat-free bone, containing significantly higher percentages of ash, calcium and phosphorus. On the other hand, compared with the low-calcium diet, it depressed the total retention of fluorine by 10.5% for the smaller rats, and 12.8% for the larger rats. While quantitatively most of this depression of fluorine retention occurred in the bones, the percentage depressions were more marked in the teeth and in the soft tissues. The percentage decreases in fluorine content for the

TABLE I  
The effects of low and high levels of dietary calcium on growing rats, with particular reference to the disposition of the dietary fluorine.

	DATA FOR 8 PAIRS OF SMALLER RATS						DATA FOR 12 PAIRS OF LARGER RATS					
	Averages		Differences between pair-mates		Probability of a fortuitous outcome		Averages		Differences between pair-mates		Probability of a fortuitous outcome	
	Low-Ca rats	High-Ca rats	Average	Standard deviation	Low-Ca rats	High-Ca rats	Average	Standard deviation	Low-Ca rats	High-Ca rats	Average	Standard deviation
Initial body weight, gm.	4.4	4.4	8.5	17.8	0.12	120	121	2.92	16.3	0.28	...	...
Gain in body weight, gm.	232	240	...	...	...	157	160	...	...	...	...	...
Final body length, mm.	221	224	...	...	...	225	228	...	...	...	...	...
Days on experiment	106	106	...	...	...	98	98	...	...	...	...	...
Food consumed, gm.	1000	1000	...	...	...	1000	1000	...	...	...	...	...
F consumed, mg.	9.40	9.42	...	...	...	9.40	9.42	...	...	...	...	...
Dry weight of fat-free bones, gm.	8.030	12.104	Highly significant	...	...	10.176	12.075	Highly significant	...	...	...	...
F in bones, p.p.m.	460	280	180	41.8	<0.00005	403	301	102	36	<0.00001	0.0001	0.0001
F in bones, mg.	3.692	3.366	0.326	0.298	0.012	4.103	3.633	0.470	0.283	0.0001	...	...
Dry weight of teeth, gm.	0.4489	0.4618	0.129	0.370	0.19	0.4651	0.4660	...	...	...	...	...
F in teeth, p.p.m.	232	171	61.4	21.5	0.00007	206	165	41.2	27.3	0.0002	0.0002	0.0002
F in teeth, mg.	0.104	0.079	0.0255	0.0141	0.0010	0.096	0.077	0.0197	0.0127	0.0002	0.0002	0.0002
Flesh weight of tissue, gm.	260	260	...	...	...	257	257	...	...	...	...	...
F in soft tissues, p.p.m.	0.70	0.54	0.159	0.103	0.0024	0.60	0.60	...	...	...	...	...
F in soft tissues, mg.	0.181	0.138	0.0429	0.0377	0.0098	0.151	0.148	0.0206 <sup>a</sup>	0.0202	0.016	0.016	0.016
Empty carcass weight, gm.	276	284	...	...	...	277	281	...	...	...	...	...
F in carcass, mg.	3.976	3.583	...	...	...	4.351	3.857	...	...	...	...	...
F retention, <sup>1</sup> mg.	3.738	3.346	0.392	0.319	0.007	3.857	3.362	0.439	2.70	<0.00001	...	...
F retention, pct.	39.77	35.52	...	...	...	44.63	38.98	...	...	...	...	...
Ash in dry bones, pct.	42.85	49.85	7.00	3.51	0.0006	46.92	49.99	3.07	3.61	0.0086	...	...
Ca in dry bones, pct.	15.54	18.55	3.01	1.44	0.0005	17.23	18.65	1.42	1.39	0.0031	0.0031	0.0031
P in dry bones, pct.	8.26	9.35	1.09	0.680	0.0019	8.82	9.29	0.474	0.707	0.024	0.024	0.024
Ca:P ratio in bones	1.88	1.98	...	...	...	1.95	2.01	...	...	...	...	...
Ca:F ratio in bones	340	670	...	...	...	430	626	...	...	...	...	...
P:F ratio in bones	181	338	...	...	...	220	312	...	...	...	...	...

<sup>a</sup> The initial fluorine content of check rats was found to be 5.4 p.p.m. of live weight for the smaller rats and 4.1 p.p.m. for the larger.

<sup>1</sup> After eliminating one aberrant difference by means of Chauvenet's criterion.

smaller and the larger rats were, respectively, 8.8 and 11.5 for the bones, 24.5 and 20.5 for the teeth, and 23.7 and 13.6 for the soft tissues.

From these values one may infer that the protective effect of an increase in the calcium content of the diet from an inadequate (0.23%) to an adequate (0.73%) level is greater than the total depression of fluorine retention would indicate, since the most harmful effects of fluorine are presumably exerted on the teeth and on the soft, or protoplasmic, tissues.

Also the average values cited above would indicate that the protective action of calcium is greater with respect to the teeth and the soft tissues for the younger rats than for the older. However, a statistical analysis of the two sets of data failed to reveal conclusive evidence to that effect, although the evidence was quite indicative ( $P = 0.075$ ) with reference to the soft tissues.

*The effect of variable intakes of dietary phosphorus on fluorine retention.* In experiments 2 and 3, the low-phosphorus diets were poorly consumed and the growth of all trios was consequently very slow. On equal intakes of food, the rats on the two higher levels of phosphorus exhibited significantly greater gains in body weight and attained significantly greater body lengths. Also, in experiment 3, the high-phosphorus rats gained in weight significantly more than the medium-phosphorus rats and attained significantly greater body lengths.

However, the results of the carcass analyses in these two experiments were largely negative in significance. Although the amounts of fluorine retained averaged higher for the low-phosphorus rats than for the medium-phosphorus rats in both experiments, the differences were statistically quite insignificant, the probability of a fortuitous outcome being 0.33 in experiment 2 and 0.28 in experiment 3. The differences between the medium- and high-phosphorus rats were also clearly insignificant. An average of 36.6% of the fluorine intake was retained in experiment 2, and 30.7% in experiment 3. The total fluorine contents of the bones, teeth and soft tissues also

were not significantly affected by the variable intake of dietary phosphorus in both experiments.

In both experiments, the average weight of dry fat-free bones was greater by about 14% in the medium-phosphorus rats than in the low-phosphorus rats and the difference was highly significant. In experiment 3, but not in experiment 2, the weight of dry fat-free skeleton increased significantly by about 7% in the high-phosphorus rats as compared with their pair-mates on the medium-phosphorus diet.

Since the total fluorine content of the skeleton was not significantly affected by the concentration of phosphorus in the diet, while the weight of dry fat-free skeleton was affected, the concentration of fluorine in the bone was modified by the variable phosphorus intake. In experiment 2, in which a higher level of fluorine was fed, the concentration of fluorine in the dry fat-free bone averaged 793, 687, and 662 p.p.m., respectively, for the low-, medium-, and high-phosphorus rats. Only the difference between the low- and the medium-phosphorus rats is significant. In experiment 3, the average concentrations in the three groups of rats were 378, 322, and 299, respectively, and both differences between successive averages are significant.

Evidently, within the range studied, variation in the concentration of phosphorus in the diet does not appreciably modify the assimilation of low levels of dietary fluorine.

#### SUMMARY AND CONCLUSIONS

In three experiments, involving a total of seventy-six rats, the effects of varying levels of dietary calcium, dietary phosphorus remaining constant, and of dietary phosphorus, dietary calcium remaining constant, on the retention and distribution of low levels of dietary fluorine (9, 12 and 32 p.p.m.) among skeleton, teeth and soft tissues were determined. The experiments involved equalized feeding of rats on comparable rations, analysis of check rats at the start of the experiments and analysis of all experimental rats at the termination of feed-

ing periods during which 1000 gm., 500 gm., and 600 gm. of food were consumed, respectively, per rat. The results secured warrant the following conclusions:

1. An increase in the concentration of dietary calcium from 0.23 to 0.73%, phosphorus remaining constant, produces a distinctly heavier dry fat-free skeleton, containing significantly higher percentages of ash, calcium and phosphorus in the growing rat. On the other hand, it depresses the total retention of fluorine by 10 to 13%, and to a greater extent the deposition of fluorine in teeth and soft tissue. Quite probably this protective action of calcium against assimilation of fluorine is greater in young than in older rats.
2. An increase in the concentration of dietary phosphorus from 0.14 to 0.71%, dietary calcium remaining constant, produces in young growing rats a greater appetite for food, a slightly greater rate of growth and a distinctly heavier dry fat-free skeleton. However, it does not modify appreciably the total retention of fluorine or the distribution of retained fluorine among bones, teeth and soft tissue. The increased weight of skeleton with no increase in its content of fluorine, does, however, depress the concentration of fluorine in the dry fat-free bones.
3. Presumably dietary calcium consumed in concentrations above certain minimum (inadequate ?) levels, protects the body against dietary fluorine, in food or in water, by impairing its assimilation to some extent, especially in those tissues, the teeth and the soft tissues, where its most deleterious effects would be exerted.

#### LITERATURE CITED

- CARTER, R. H., AND R. L. BUSBEY 1939 The use of fluorine compounds as insecticides, a review with annotated bibliography. U. S. Dept. Agr. Bur. Entomol. Plant Quarantine E-466, p. 156.
- DE EDs, FLOYD 1933 Chronic fluorine intoxication. A review. Medicine, vol. 12, pp. 1-60.
- HAUCK, H. M., H. STEENBOCK AND H. T. PARSONS 1933 The effect of the level of calcium intake on the calcification of bones and teeth during fluorine toxicosis. Am. J. Physiol., vol. 103, pp. 489-493.

- LAWRENZ, M., H. H. MITCHELL AND W. A. RUTH 1939 a The comparative toxicity of fluorine in calcium fluoride and in cryolite. *J. Nutrition*, vol. 18, pp. 115-125.
- 1939 b A comparison of the toxicity of fluorine in the form of cryolite administered in water and in food. *J. Nutrition*, vol. 18, pp. 127-141.
- MACHLE, WILLARD, AND E. E. EVANS 1940 Exposure to fluorine in industry. *J. Ind. Hyg. Tox.*, vol. 22, pp. 213-217.
- McCLURE, F. J. 1939 Fluorides in food and drinking water. A comparison of effects of water-ingested versus food-ingested sodium fluoride. *National Inst. Health Bul.* 172, pp. 53.
- NICHOLS, M. S. 1939 Occurrence, pathological aspects and treatment of fluoride waters. *Am. J. Public Health*, vol. 29, pp. 991-998.
- PIERLE, C. A. 1926 Production of mottling and brown stain. *J. Am. Dent. Assoc.*, vol. 13, p. 999.
- SCHULZ, J. A. 1938 Fluorine toxicosis in the albino rat. *Iowa Agr. Exp. Sta. Res. Bul.* 247, pp. 165-242.
- SHIELDS, J. B., AND H. H. MITCHELL 1941 The effect of calcium and phosphorus on the metabolism of lead. (In press.)
- SMITH, MARGARET CAMMACK 1935-1936 Dietary factors in relation to mottled enamel. *J. Dental Res.*, vol. XV, pp. 281-290.
- STUDENT 1925 New tables for testing the significance of observations. *Metron*, vol. 5, pp. 105-120.



# THE EXCRETION OF SELENIUM BY RATS ON A SELENIFEROUS WHEAT RATION<sup>1</sup>

H. D. ANDERSON AND A. L. MOXON

*South Dakota Agricultural Experiment Station, Brookings*

TWO FIGURES

(Received for publication February 14, 1941)

Students of public health frequently raise the question whether animals which have subsisted on seleniferous rations are safe for human consumption. The answer can hardly be forthcoming until more is known of the actual effects of selenium on man. At present, outside of certain skin lesions (Lemley, '40) due to selenium sensitivity, little is known of the human pathology due to the ingestion of selenium as it may occur in foods.

With the above question in mind, it becomes of interest to determine how much selenium is stored by the animal and how fast and by what means this element is excreted.

In this laboratory Moxon ('41) observed that when rats were fed a toxic wheat ration containing 18 p.p.m. of selenium, the fecal excretion of selenium increased from 16% to 22% of the ingested selenium over a period of 6 weeks. The amount excreted in the urine increased from 20.2% to 35.5% of the selenium ingested. Thus the animals retained 63.5% of the selenium intake during the first week. The percentage of retained selenium gradually but steadily decreased until the sixth week at which time it amounted to but 40%. This continual decrease in selenium retention, signifying an approach to an equilibrium state, suggested that a saturation

<sup>1</sup>Approved for publication by the director of the South Dakota Agricultural Experiment Station as journal series 135.

process was involved. The point of equilibrium or saturation was not reached in this particular experiment because the animals invariably died first.

Inorganic selenium compounds, when injected or administered orally, have been shown to be excreted quite rapidly by cats, rabbits (Smith et al., '37) and rats (Gortner and Lewis, '39); most of the selenium was eliminated within 2 weeks after the administration was discontinued. Smith et al. ('38) reported a greater retention of the naturally occurring organic selenium compounds than of  $\text{Na}_2\text{SeO}_3$ . In our experiments no such difference in retention of natural selenium compounds and  $\text{Na}_2\text{SeO}_3$  was observed but our level of selenium supply was considerably lower (18 p.p.m.).

Munsell et al. ('36), in some preliminary studies on the retention of naturally occurring selenium compounds, indicated that there is a rather slow elimination of the selenium by the rat, but she did not establish how rapidly the major portions of this excretion took place, i.e., whether it was a constant amount or subject to considerable change. From a consumer's standpoint it should be interesting to know how fast and to what extent most of the elimination takes place. The following experiment was designed to supply some of this information.

#### EXPERIMENT

Previous data, obtained in this laboratory, indicated that the selenium concentration of the rats on a seleniferous diet showed a sufficiently close correlation within the groups to justify sacrificing a few animals from time to time as representatives of the group. In this experiment, then, we classified the animals into equivalent groups according to weight, sex and age, placed them on the seleniferous ration, and analyzed representative animals weekly as an index of the status of the whole group.

In the first series thirty adult rats received a ration having the following percentage composition: wheat no. 607 (30 p.p.m. of Se) 82; casein 10; salt mixture (McCullum) 1; pure

leaf lard 3; dried yeast<sup>2</sup> 2; and cod liver oil<sup>3</sup> 2. This ration contained approximately 25 p.p.m. of selenium and was fed ad libitum for a period of 4 weeks. The animals were then transferred to the stock ration having the following percentage composition: corn 32.5; wheat 37.5; rolled oats 20.0; alfalfa meal 5; and 1% each of cod liver oil, yeast,  $\text{Ca}_3(\text{PO}_4)_2$ , NaCl and  $\text{CaCO}_3$  respectively.

Each week of the experiment, three representative animals were killed by decapitation and autopsied. Before being killed, the animals were brushed carefully to remove any adhering food particles. The livers were taken as one sample for selenium analysis, the rest of the carcass serving as another. After decapitation the liver was removed, washed in distilled water, drained on a pad of clean filter paper and dried on a watch glass. The alimentary tract was removed, split lengthwise, washed free of undigested food, and returned to the carcass. The whole carcass was then ground in a meat chopper and dried on a large watch glass. Analyses were made by the modified method of Robinson, Dudley, Williams and Byers as described in a previous report (Moxon, '37).

The second series was designed to be a repetition of the first but this time young growing rats were used instead of adult animals. Thirty rats weighing approximately 50 gm. each received the toxic ration for the 4-week period and were then transferred to the stock ration. A control group of ten rats was included in this series to serve as a basis for comparing the growth response.

The data presented graphically in figure 1 indicate that the animals continued to store selenium throughout most of the 4-week period, the amount stored reaching a maximum at the time that the toxic ration was removed. The young rats apparently stored greater amounts in their body tissues than

<sup>2</sup> Yeast foam powder manufactured by the Northwestern Yeast Co., Chicago, Ill.

<sup>3</sup> Cod liver oil containing 80 I.U. of vitamin D per gram, obtained from the National Oil Products Co., Harrison, N. J.

the adults, although the liver storage in the two groups proved to be roughly comparable. After the rats were transferred to the non-seleniferous stock ration there was a rapid fall in the concentration of selenium, especially in the livers. The quantity in this organ did not reach zero, however, and this may be taken as indicating that small amounts are bound too securely to be eliminated over a period of 6 weeks.

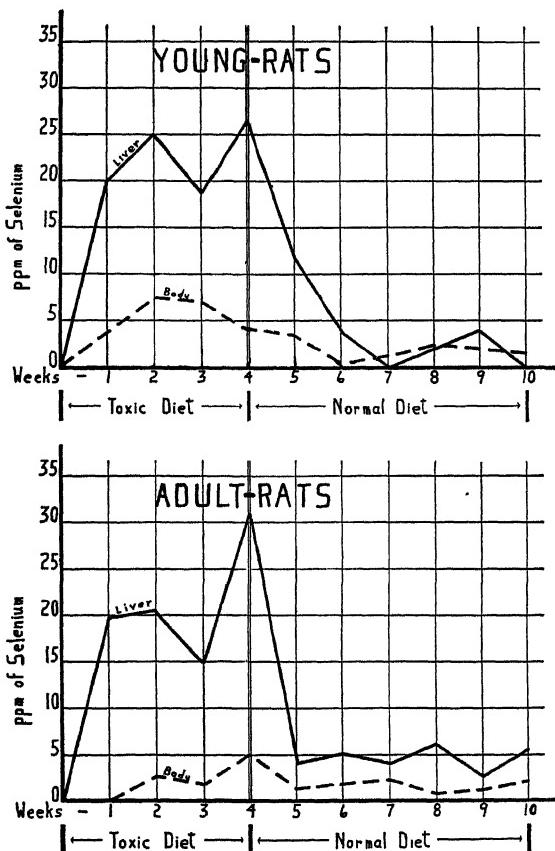


Fig. 1 Curves showing the concentration of selenium in the livers and the rest of the carcass of rats on the toxic diet, and the results obtained when animals are transferred to non-toxic rations.

Each point represents a composite sample from three rats, since the livers of individual rats offer too small a sample for accurate analysis.

The growth responses to the toxic and non-toxic ration are shown in figure 2. Subsistence on the toxic ration for 4 weeks had a considerably depressant effect upon the body-weight of both the young and adult rats.

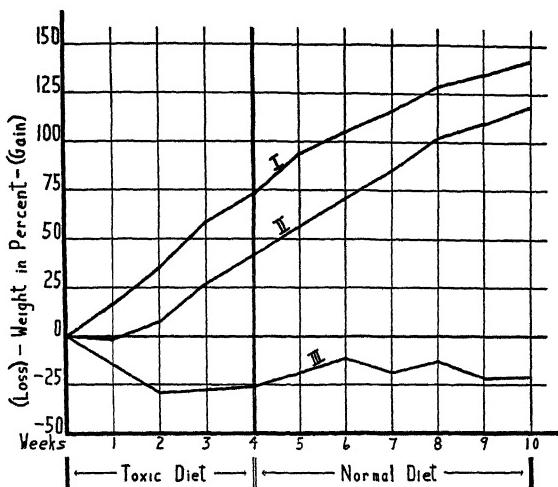


Fig. 2 Curves showing the percentage gain or loss in body weight of rats on the toxic rations as compared with the control group. (I) refers to the control group, (II) the young rats on the toxic diet and (III) the adult rats on the toxic diet.

#### DISCUSSION

The results obtained in this experiment indicate that at least for the rat, most of the selenium is eliminated within 2 weeks after the animals are transferred from a seleniferous wheat ration to a selenium-free diet. Some appears to be retained however, and this would account for the continued excretion of selenium which has been observed in cats (Smith et al., '38) to extend over a period of several months. If this condition exists in other species, then in spite of the fact that some of the naturally occurring selenium is retained for several months by the animal, from a quantitative standpoint much could be gained by placing the animals on a non-seleniferous ration for an appropriate period before slaughtering them. At least a large part of the selenium could be prevented from entering the human diet by this procedure.

The recent observation that bromobenzene (Moxon et al., '40) increases the selenium excretion from the animal body offers another possible procedure for making selenized animals safer for human consumption. Not enough is known as yet of the action of this compound in lowering the tissue concentration of selenium to warrant specification of details of such a method at the present time, but the observation opens a field worthy of more extensive investigation.

#### SUMMARY

1. The greater portion of the selenium absorbed by rats from naturally occurring seleniferous wheat was excreted when the animal was transferred to a non-seleniferous stock ration. Some of the selenium was eliminated very slowly and appeared to be bound by the body tissues but the greater portion of the selenium appeared to be eliminated within 2 weeks.
2. Young rats appeared to store more selenium in the body tissues other than liver than old rats. Storage in the livers appeared to be similar.
3. Feeding of the toxic ration for only 4 weeks had a depressing effect upon the body weight of both young and adult rats.

#### LITERATURE CITED

- GORTNER, R. A., JR., AND H. B. LEWIS 1939 The retention and excretion of selenium after the administration of sodium-selenite to white rats. *J. Pharm. Exp. Ther.*, vol. 67, p. 358.
- LEMLEY, RAY E. 1940 Selenium poisoning in the human. *The Journal Lanceet*, Minneapolis, vol. 60, p. 528.
- MOXON, A. L. 1937 Alkali disease or selenium poisoning. *S. Dak. Agric. Expt. Station Bull.* 311, p. 62.
- 1941 Unpublished data.
- MOXON, A. L., A. E. SCHAEFER, H. A. LARDY, K. P. DUBOIS AND O. E. OLSON 1940 Increasing the rate of excretion of selenium from selenized animals by the administration of bromobenzene. *J. Biol. Chem.*, vol. 132, p. 785.
- MUNSELL, H. E., G. M. DEVANEY AND M. H. KENNEDY 1936 Toxicity of food containing selenium as shown by its effect on the rat. *U. S. Dept. Agric. Techn. Bull.* 543, p. 21.
- SMITH, M. I., B. B. WESTFALL AND E. F. STOHLMAN, JR. 1937 The elimination of selenium and its distribution in the tissues. *U. S. Pub. Health Repts.*, vol. 52, p. 1171.
- 1938 Studies on the fate of selenium in the organism. *U. S. Pub. Health Repts.*, vol. 53, p. 1199.

# IMPROVED DIETS FOR NUTRITIONAL AND PATHOLOGIC STUDIES OF CHOLINE DEFICIENCY IN YOUNG RATS

R. W. ENGEL AND W. D. SALMON

*Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn*

TWO PLATES (FIVE FIGURES)

(Received for publication February 17, 1941)

## INTRODUCTION

In the investigations of vitamin B<sub>6</sub> in this laboratory symptoms of severe toxicosis frequently developed in rats fed purified diets supplemented with crystalline vitamins. The work of Griffith and Wade ('39 a) suggested that this toxicosis was a result of choline deficiency.

It is the purpose of this paper to report the effectiveness of various purified diets in producing choline-deficiency symptoms and to report the pathological changes accompanying these symptoms.

## MATERIALS AND METHODS

*Animals.* Twenty-three-day-old male and female rats of the Steenbock and Wistar strains were kept in individual cages with raised screen floors. About 300 animals were used in these studies.

*Diets.* The diets listed in table 1 were used with varying degrees of success. The animals had continuous access to the diet except for a few hours each day when supplements were fed. Each animal received daily, in separate food jars, 20 µg.

each of thiamine chloride,<sup>1</sup> pyridoxine, and riboflavin, factor 2 concentrate equivalent to 1 gm. of liver and, in the case of rats on the fat-low diets, 0.1 ml. of corn oil. Vitamins A and D were supplied weekly in the form of beta-carotene and calciferol. Control animals each received 10 to 20 mg. of choline chloride per day.

TABLE I  
*Percentage composition of the diets*

DIET NO.	31 P	31 CP	31 PM	31 PMC	31 CPMC	31 F <sub>2</sub>	6 L
Arachin <sup>1</sup>	18	17.7					
Peanut meal <sup>2</sup>			24	30	29.7		
Casein <sup>3</sup>				6	6	18	17.7
Sucrose	78	78	72	54	54	78	68
Salt <sup>4</sup>	4	4	4	4	4	4	4
Lard				6	6		10
Cystine		0.3			0.3		0.3

<sup>1</sup> Prepared from peanut meal according to Johns et al. ('16).

<sup>2</sup> Percolated with 61.5% alcohol by volume for 24 hours, followed by five 2-hour extractions with boiling 95% alcohol.

<sup>3</sup> Labco casein extracted four times (2 hours each) with boiling 95% alcohol.

<sup>4</sup> J. Biol. Chem., 1930, vol. 89, p. 199.

*Technical procedures.* The animals were killed by bleeding through the carotid vessels. Materials desired for routine microscopy were preserved in Bouin's fluid, embedded in paraffin and stained with hematoxylin and eosin. In some cases formalin-fixed sections of liver were sectioned in the frozen state and stained with scarlet red. Liver, kidney, and adrenal tissues were treated with Maximov's osmic acid stain for fat; the xanthydrol reaction (Romeis, '28) was used on

<sup>1</sup> The crystalline vitamins were generously supplied by Merck and Company, Rahway, N. J. The pantothenic acid fraction (factor 2 concentrate) used in this work was prepared as follows: 500 gm. of liver extract (Lilly no. 343) in 1500 ml. of H<sub>2</sub>O was treated with 500 gm. of English fullers earth which was filtered out and washed with five 500 ml. portions of H<sub>2</sub>O. The treatment was repeated five times with 250 gm. of fullers earth, the volume of the filtrate being kept up to 1 liter. The filtrate and washings were concentrated to 1250 ml., filtered and stored under Skelly Solve B at 3–5°C. until fed. In later experiments, it has been found that identical results are obtained by substituting calcium pantothenate (Merck) for the liver preparation.

some kidney and brain sections; and some kidney sections were treated with Mallory's ('38) aniline blue collagen stain.

#### EXPERIMENTAL RESULTS

##### *Effectiveness of the diets*

Diets 31 P, 31 CP, and 31 PM were equally effective in producing severe choline deficiency in all the animals. However, these diets were obviously inadequate in other essential food factors since control animals receiving 10 mg. daily of choline chloride made weekly gains of only about 5 gm. Diet 31 PMC produced severe symptoms in all the animals in 6 to 10 days. Control animals (20 mg. of choline chloride daily) remained normal and consistently gained about 3 gm. daily. Table 2 presents growth records of rats receiving this diet. Cystine added to this diet failed to stimulate growth.

TABLE 2  
*Weight and mortality record of rats fed diets with and without choline*

DIET NO.	AVERAGE INITIAL WEIGHT	AVERAGE BODY WEIGHT GAIN IN GRAMS IN					
		6 days	7 days	8 days	9 days	10 days	14 days
gm.							
	(20)	(20)	(20)	(18)	(16)	(11)	(1)
31 PMC	56	10	12	11	9	4	2
31 PMC+	(20)	(20)	(20)	(20)	(20)	(20)	(20)
20 mg. choline daily	54	18	22	25	29	34	48
31 CPMC +	(8)	(8)	(8)	(8)	(8)	(8)	(8)
20 mg. choline daily	49	15	20	23	28	33	49

The figures in parentheses indicate the number of rats surviving.

Diet 6 L produced gains of 2 to 3 gm. daily, but resulted in severe, acute symptoms in only about two-thirds of the animals. Diet 31 F<sub>2</sub> was used routinely in investigations concerning vitamin B-complex and fat deficiencies. On this diet about 50% of the male animals developed the typical symptoms and about half of these died. Females were somewhat less susceptible than the males on this diet. Considerable variations were also noted between different litters. Such litter or sex dif-

ferences were not noticed when the diets containing peanut meal were used.

It should be emphasized that the animals which made the best gains during the first 5 or 6 days on experiment usually developed severe symptoms earliest and failed most rapidly.

#### *Description of the symptoms*

Choline deficiency symptoms usually appeared within 6 to 10 days after the animals were placed on the experimental diets. Concomitant with drowsiness and inactivity, palpably enlarged kidneys and abdominal distention occurred. In severe, acute cases death resulted within 48 hours after the appearance of the first symptoms. Death was preceded by several minutes of extremely labored breathing, tremors, and coma. Extreme pain was evidenced by the fact that occasionally animals succumbed with their jaws set firmly in the cage wire. Circulatory impairment was indicated in the final stages by the loss of normal skin color, and lowered temperatures, particularly noticeable in the extremities.

Less common symptoms were diarrhea and eye hemorrhage. The eye hemorrhage appeared to originate in the ciliary vessels and spread into the posterior chamber. In a few instances the animals exhibited vertigo.

Blood samples from four rats on diet 31 PMC were pooled and the non-protein nitrogen was determined and found to be 247 mg./100 ml. of blood. Pooled samples of blood from two controls (20 mg. of choline chloride) had a non-protein nitrogen of 42 mg./100 ml.

#### *Necropsy observations*

Constant findings were enlarged, firm, bright red, hemorrhagic kidneys with thickened capsule, extremely pale, fatty livers (table 3), and atrophied thymus. The kidney weights of the most severe cases averaged over 100% more than the kidney weights of control animals fed the same diet plus choline. Hydrothorax was present in most cases, and less

TABLE 3

*Kidney weight and liver fat in rats fed diets with and without choline*

DIET NO.	NUMBER OF RATS	FRESH KIDNEY WEIGHT IN PER CENT OF MAXIMUM BODY WEIGHT	LIVER FAT <sup>1</sup>
31 P	7	3.11	46.4
31 P + 10 mg. choline daily	8	1.33	15.6
31 CP	3	2.89	47.4
31 CP + 10 mg. choline daily	4	1.31	32.3
31 PM	4	2.68	45.6
31 PM + 10 mg. choline daily	4	1.32	24.0
31 PMC	4	2.61	49.5
31 PMC + 10 mg. choline daily	3	1.40	25.9
6 L	8	2.37	55.1
6 L + 20 mg. choline daily	8	1.14	11.4
31 F <sub>2</sub>	8	2.37	...

<sup>1</sup> Per cent ether extract in the dry liver.

frequently, ascites was found. Foci of hemorrhage in the heart muscle, adrenal cortex, and lungs were frequently present. In many animals the lumbar and sacral lymph nodes were filled with blood. The spleen varied in appearance: in some animals it was extremely swollen and bright red; in others it was shrunken and quite pale. Severe congestion in the mesenteric vessels and in the descending aorta was also frequently seen.

Rats which survived an acute attack resumed growth and survived for from several weeks to several months. In such cases necropsy revealed a kidney surface severely pitted and scarred with connective tissue, and the capsule adhering to the surface. Animals in which the acute attack was less severe made an apparently complete recovery and showed only mild connective tissue scarring of the kidney surface.

#### *Microscopic observations*

*Kidney.* The renal hemorrhage originated in the arteriole beds in the periphery of the cortex. The efferent cortical vessels were primarily involved, but the glomeruli were also affected in the severely hemorrhagic condition. In the acute

fatal stages the kidney cortex became hemorrhagic throughout with disintegration and necrosis of the tubular system. In such kidneys the collecting tubules throughout the medulla were filled with blood, cellular debris, and hyaline masses. Extensive hemorrhage occurred between the capsule and the kidney surface. The capsule was thickened by connective tissue proliferation.

In animals which succumbed to the chronic form of the disease the tubular system of the kidney cortex was completely invaded by connective tissue with some calcification. The blood vessel walls were thickened in certain of the most severely scarred areas. The tubular system of the medulla retained its normal structure. Mild chronic cases presented only wedge-shaped areas of connective tissue invasion from the cortical surface inward, with tubules still normal in the adjoining areas.

*Liver.* The primary change in the liver was the uniform distribution of massive stores of fat in the liver cells. Occasionally the central veins were extremely distended and filled with blood.

*Thymus.* Parenchymatous atrophy and subsequent proliferation of fatty tissue resembled normal involution. Frontal sections through the organ showed only a small area of normal glandular tissue remaining.

*Spleen.* Cellular changes in the red pulp were variable. In some cases severe congestion was present and the red pulp was overcrowded with erythrocytes. In other cases the red pulp was atrophied and the whole organ shrunken. The connective tissue framework was usually normal.

*Lymph nodes.* The cortical substance sometimes was congested with erythrocytes and occasionally hemorrhage was present. Frequently, however, there were no abnormal changes.

*Adrenals.* Hemorrhage occurred in the arterioles of the capsule and between the cell columns of the cortex. It was most severe in the zona glomerulosa but occasionally it extended inward as far as the medulla. The cortical cell columns were often necrotic where severe hemorrhage had occurred.

*Lungs.* Usually severe congestion with varying degrees of edema and hemorrhage was present. Blood vessels were filled with blood and were severely distended.

*Heart.* Foci of hemorrhage and infiltration of white blood cells appeared in the myocardium in both right and left ventricles. Frequently there was extensive necrosis of the muscle fibers in the severely affected areas.

*Xanthydrol reaction.* Extensive deposits of needle-like crystals of xanthydrol in sections of brain and kidney indicated uremia.

#### DISCUSSION

The observations herein reported demonstrate conclusively that severe pathological changes result in young, growing rats fed diets deficient in choline. The pathological observations essentially confirm those reported by Christensen ('40).

Increase in kidney weight was found to be a reliable criterion for determining the effectiveness of various diets for producing choline deficiency. Approximately a 100% increase in kidney weight consistently resulted in either male or female rats fed the diets in which peanut meal was the sole or principal source of protein. This increase was about 65% in the animals fed diets containing casein as the principal source of protein.

Earlier work on kidney hemorrhage in rats fed purified diets has been adequately reviewed by Griffith and Wade ('40). Although it was concluded that cystine was the damaging agent, it is interesting that some of these workers were using diets which were low in choline. Griffith and Wade ('39 b, '40) and Griffith ('40 a) have shown that cystine added to the diet exaggerated choline deficiency symptoms and that either methionine or choline was protective. On the basis of these findings it would appear that the peanut meal diets used in the present investigations contained the sulphur-amino acids in ideal proportions for the production of choline deficiency. The sulphur-amino acid composition of these diets is not known, but the excellent growth obtained by the simple addition of choline chloride to diet 31 PMC indicated that these

amino acids were present in adequate amounts. Furthermore, the addition of cystine to these diets failed to stimulate growth.

Griffith ('40 b) has reported that male rats are more susceptible to choline deficiency than females. Sex and litter differences in susceptibility to choline deficiency were encountered in these experiments with diets containing casein as the sole protein, or in similar diets with 0.3% cystine added. Such litter and sex variations were not encountered, however, when the diets containing peanut meal were used. In experiments conducted to study mortality from choline deficiency on diet 31 PMC, seventy-nine out of eighty rats (forty females and thirty-nine males) died by the twelfth day of the experiment.

Since preliminary findings indicate that choline deficiency symptoms are in part manifested by high non-protein nitrogen in the blood, it is possible that the diets employed here would be ideal in studies concerning the production of experimental uremia.

The data presented here are of importance in relation to nutritional investigations where purified diets are employed. Subacute cases of choline deficiency could easily be overlooked since such animals appear quite normal and palpably enlarged kidneys is the only reliable symptom. Routine choline supplements to purified diets for the rat would appear essential on the basis of the present findings.

#### SUMMARY AND CONCLUSIONS

Diets were developed which consistently produced a fatal toxicity in young growing rats of either sex in from 6 to 10 days. The symptoms consisted of inactivity, abdominal distention, palpably enlarged kidneys, labored breathing, coma, and, in some cases, eye hemorrhage and vertigo.

Microscopic examination revealed severe hemorrhage in the kidney cortex, varying degrees of hemorrhage in the adrenals, lungs, myocardium and lymph nodes, massive deposits of fat in the liver cells and atrophy of the thymus. A high non-

protein nitrogen in the blood and a positive xanthidrol reaction indicated uremia.

Choline chloride (10 or 20 mg. per rat per day) prevented these symptoms. The advisability of routine choline supplements to purified diets in nutrition investigations with the rat has been emphasized.

The authors wish to thank Cornelia Stevens Flanagan for microtechnical assistance in this work.

#### LITERATURE CITED

- CHRISTENSEN, K. A. 1940 Microscopic study of the effect of choline deficiency in young rats. *J. Biol. Chem.*, vol. 133, p. xx.
- GRIFFITH, W. H., AND N. J. WADE 1939 a Some effects of choline low diets. *Proc. Soc. Exp. Biol. and Med.*, vol. 41, p. 188.
- 1939 b Relation of methionine, cystine, and choline to renal lesions occurring on low choline diets. *Ibid.*, p. 333.
- 1940 Choline metabolism. II. The interrelationship of choline, cystine, and methionine in the occurrence and prevention of hemorrhagic degeneration in young rats. *J. Biol. Chem.*, vol. 132, p. 627.
- GRIFFITH, W. H. 1940 a Choline metabolism. III. The effect of cystine, fat, and cholesterol on hemorrhagic degeneration in young rats. *J. Biol. Chem.*, vol. 132, p. 639.
- 1940 b Choline metabolism. IV. The relation of the age, weight, and sex of young rats to the occurrence of hemorrhagic degeneration on a low choline diet. *J. Nutrition*, vol. 19, p. 437.
- JOHNS, C. O., AND D. B. JONES 1916 The proteins of the peanut, *Arachis Hypogaea*. I. The globulins arachin and conarachin. *J. Biol. Chem.*, vol. 28, p. 77.
- MALLORY, F. B. 1938 Pathological Technique. W. B. Saunders Company, Philadelphia.
- ROMEIS, B. 1928 Taschenbuch der Mikroskopischen Technik. R. Oldenbourg, Munchen and Berlin.

PLATE 1

EXPLANATION OF FIGURES

1 Kidneys from litter mate rats after 7 days on diet 31 PMC (upper) and diet 31 PMC + 10 mg. choline chloride daily (lower). The dark surface of the upper kidney is due to hemorrhage in the cortex. Also notice the size difference.  $\times 2.5$ .

2 Choline deficiency. Section of kidney cortex. Notice the accumulation of blood in the spaces surrounding the necrotic convoluted tubules, and in the glomeruli.  $\times 150$ .

3 Higher magnification of the outlined area in figure 2. Notice the extreme congestion in Bowman's capsule and the rupture of its wall, also the necrotic tubules surrounded by the red blood cells.  $\times 320$ .

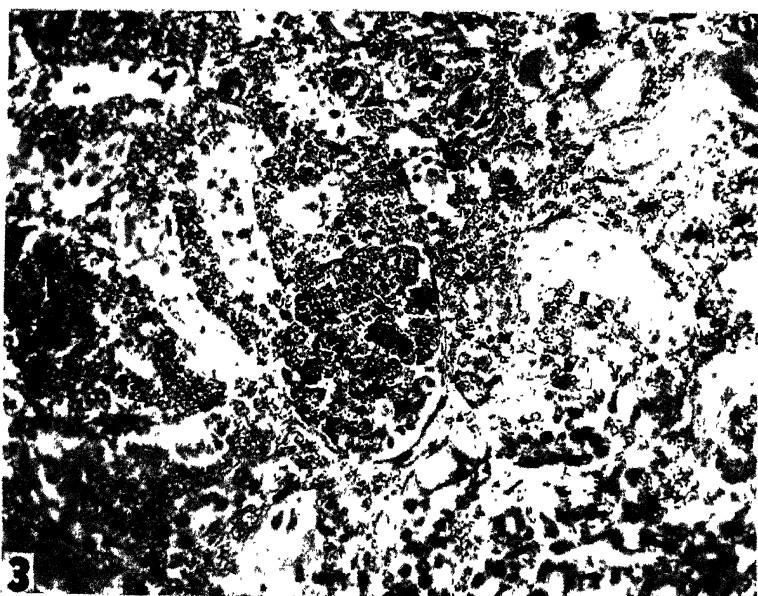
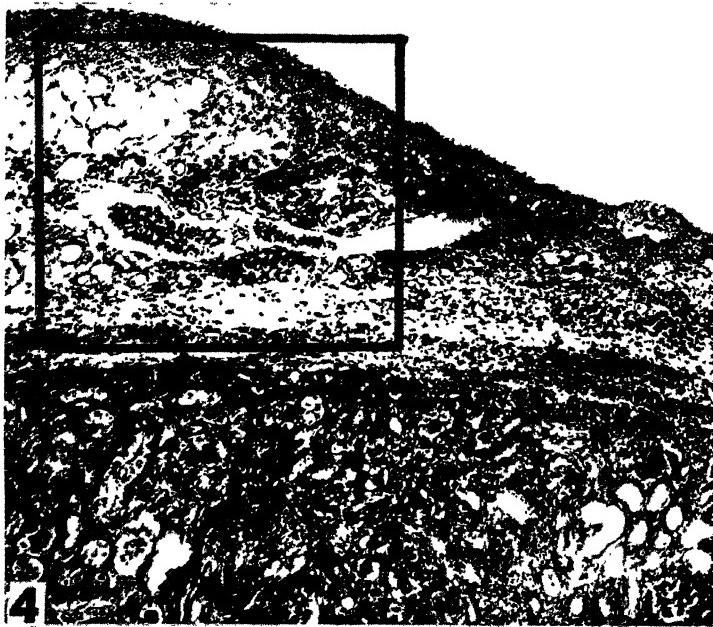


PLATE 2

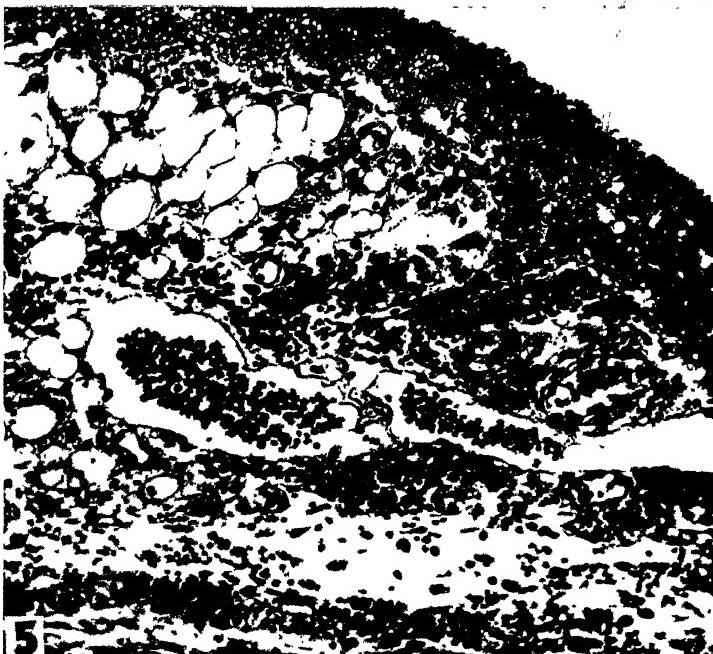
EXPLANATION OF FIGURES

4 Choline deficiency. Section of kidney capsule (upper half) and kidney cortex (lower half). Notice the extremely thickened capsule. The convoluted tubules in the cortex are completely destroyed.  $\times 115$ .

5 Higher magnification of the outlined area in figure 4. Notice the unusually large blood vessels in the capsular connective tissue and the accumulation of red blood cells in the extravascular tissue.  $\times 265$ .



4



5



# THE GROWTH CURVE OF THE ALBINO RAT IN RELATION TO DIET

THEODORE F. ZUCKER, LILIAN HALL, MARGARET YOUNG  
AND LOIS ZUCKER

*Department of Pathology, College of Physicians and Surgeons,  
Columbia University, New York*

FOUR FIGURES

(Received for publication January 28, 1941)

Studies in nutrition as carried out on laboratory animals, especially rats, have dealt largely with deficiency states. Deficiencies manifest themselves either in more or less specific symptoms or in a growth deficit below the normal growth for the given age. Deficits in growth can be measured against controls but are more frequently established by growth resumption when the deficiency is remedied. As our knowledge progresses and the requirements of experimental work become more rigorous the question often arises as a practical issue: "What is normal or optimal growth or what should be the course of growth in the rat strain employed, when there are no deficiencies?" Control groups of sizes commonly employed usually give the answer but do not always completely satisfy all the requirements. The collection of data and their analysis here presented were not undertaken with a view to the general biology of growth but arose out of the practical necessity of knowing more of the growth performance of the rat.

## EXPERIMENTAL

In table 1 will be found the mean weights from 4 weeks (age of weaning) to 17 weeks of all the males and females introduced into our breeding colony in 1936 and 1937, together

with the standard deviations and coefficients of variation. In figure 1, curve 2 represents the 1936 females, with some additional data up to 50 weeks of age and on pre-weaning growth. The individual points in the 17-50 week period are based on small numbers, and ages as well as weights are averaged, since during this period the females were bred repeatedly and were weighed only occasionally between pregnancies. We have only a few pre-weaning data on these 1936 and 1937 rats. More extensive recent data show that pre-weaning growth in our colony is properly represented by these few earlier data. The number of young each mother was allowed to raise ranged

TABLE 1  
*Body weight and variability of breeding animals on stock diet,  
1936 and 1937 combined.*

WEEKS	FEMALES N = 311			MALES N = 42		
	M	$\sigma$	CV	M	$\sigma$	CV
4	51.4	4.5	8.8	61.0	5.2	8.5
5	72.8	6.7	9.2	87.6	8.1	9.3
6	92.4	9.6	10.4	115.4	13.2	11.4
7	107.0	11.3	10.5	138.6	18.3	13.2
8	119.0	13.1	11.0	164.1	22.9	14.0
9	130.0	14.4	11.1	185.9	24.0	12.9
10	140.0	14.9	10.6	204.8	24.5	12.0
11	148.7	14.9	10.0	221.3	23.9	10.8
12	156.9	14.8	9.6	236.6	23.2	9.8
13	163.0	14.8	9.1	249.6	23.8	9.5
14	168.9	15.5	9.2	261.7	24.4	9.3
15	173.2	15.1	8.7	271.4	25.9	9.5
16	178.5	17.2	9.5	280.6	25.8	9.2
17	182.8	18.3	10.0	288.1	27.9	9.7

$$\sigma = \sqrt{\frac{\sum d^2}{N-1}}$$

$$CV = \frac{\sigma}{M} \times 100.$$

The mean values (M) were obtained for each age on the same 311 females and the same 42 males. The age tabulated is the actual age to within a day of each individual rat. Weights were determined to the nearest gram.

The standard deviations ( $\sigma$ ) and coefficients of variation (CV) are intended to characterize these particular data only. They do not characterize the colony as a whole because the breeding stock animals are selected at weaning so that the weights cluster around chosen means. The coefficients of variation of the weaning weights of the total (unselected) population during 1936 are: male CV = 15.6, N = 603; female CV = 14.5, N = 676. We do not know whether the peak in variability at about 8 weeks, with males more variable than females, found for the breeding stock would also be found for the total colony output (see Sherman and Campbell, '34).

from four to eight, seven being by far the most frequent, 75% of the total being either six or seven. The animals were fed upon our regular stock diet, with no special supplements at any time. This diet contains 15% each of ground yellow corn, ground hulled barley, ground shelled oats, ground whole wheat and soy bean meal. It contains 10% each of meat scrap and whole milk powder, 2% each of alfalfa leaf meal and sodium chloride, and 0.5% each of calcium carbonate and molasses (as a binder in pellet making). Essentially this form of diet has been in use in this laboratory since 1932. In 1936 it became available in pellet form, and has been found convenient and satisfactory in our own colony as well as in several others in this institution. The subject matter of table 2 discussed below indicates that the stock diet as far as growth is concerned has no properties different from the diets of the other investigators there mentioned.

#### DISCUSSION

*Formulation of data.* Figure 1 is a comparison of our growth curve with several others in the literature. Curve 1 represents the growth of female rats of The Wistar Institute colony; instead of using the smoothed curve of Donaldson's monograph ('24) we have taken the original data which appear only in the paper of Donaldson, Dunn and Watson ('06). For a time this was considered standard rat growth and was used by Robertson, Brody, and Pearl as the basis for equations in the theoretical study of growth. Its salient characteristic is a sigmoid form between the ages of weaning and adult life, and it was this feature, with its point of inflection located in the post-weaning period as shown in the graph, around which the theoretical considerations largely centered. Referring to these generalizations, in presenting the results of improved diets Osborne and Mendel ('26) say: "The 'curves of growth' . . . may not be in harmony with some of the current interpretations of growth." Their 1926 data were nearly free of the post-weaning point of inflection, and what is left of it occurs too early to fit in with the interpretations mentioned. Curve 3

of figure 1 represents the most recent growth curve of the Osborne-Mendel colony (Mendel and Hubbell, '35).

Curves 2 and 3 are representative of the greatly simplified shape of post-weaning rat growth obtained on the newer stock diets. All growth curves have a point of inflection, but this is now completely transferred to the pre-weaning period as illustrated in figure 1. The simple form, with constantly decreasing slope, with no trace remaining of the strongly marked sigmoid

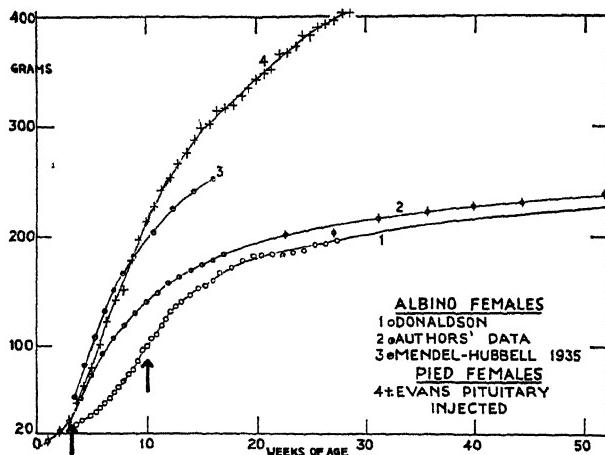


Fig. 1 Rat growth curves—ordinary (linear) plotting. Authors' data represent 167 female rats reared for breeding purposes in 1936. Arrows indicate the point of inflection in the Donaldson data, at 10 weeks, and in the authors' data, at 3 weeks. The point of inflection is the point where the curve is steepest.

form of the older data, seemed to justify the attempt to transform it into a straight line function of variables based on time and weight. This was done simply for greater ease of interpretation of data and comparison of results. Such an empirical transformation, if it leads to a simple equation, is always useful but need not have, as such, any reference to theoretical interpretation or to the mechanism of the growth process.

It was found that, if the logarithm of the weight is plotted against the reciprocal of time, a good straight line results. The formula for this straight line is:

$$\log W = \frac{-k}{t} + \log A,$$

$W$  being the weight at time  $t$ . The equation is a very simple one, containing merely the minimum number of constants necessary to define a straight line;  $A$  is the weight approached asymptotically<sup>1</sup> in the adult animal ( $\log A$  being the intercept of the straight line), and  $k$  is the slope of the line which characterizes the rate of growth ( $k = \frac{\log W_2 - \log W_1}{1/t_2 - 1/t_1}$ ). On semi-log paper with a time axis of reciprocals (4 weeks = 0.25, 5 weeks = 0.20,

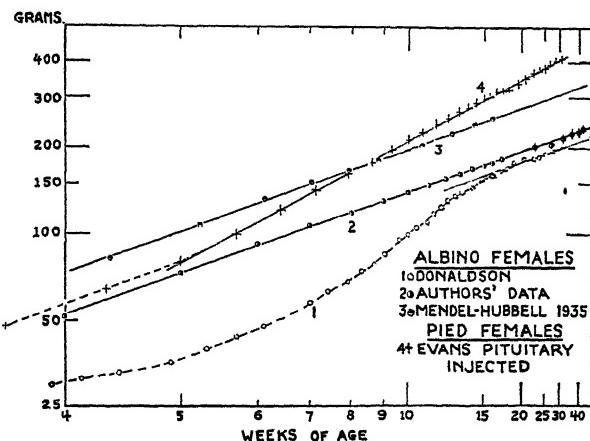


Fig. 2 Log-reciprocal plotting (logarithm of  $W$  against reciprocal of time) of the data of figure 1. For clarity the proportions of this graph resemble those of figure 1, and background lines are omitted.

etc.) the data can be plotted directly as obtained, with no preliminary calculations or adjustment of constants as is the case in the application of most other proposed equations, and of all other proposed equations covering this time range. Figure 2 is such a plot of the data of figure 1. Figure 3 shows our 1937 female data, and the averaged 1936 and 1937 data on males,

<sup>1</sup> The asymptotic growth formula may at first sight appear disturbing. It is however by no means irrational particularly in the rat where closure of the epiphyses of the long bones happens very late in life (Dawson, '25), if it ever happens so completely that further growth in length is excluded. Due consideration should be given to the fact (McCay et al., '39) that after 1000 days of retarded growth, rats are still able to resume growth. Because of the nearly universal respiratory infections after middle life, it is difficult to obtain valid data to prove or disprove asymptotic growth.

plotted on the log-reciprocal paper used routinely in our laboratory. Both with our data and with those of Mendel and Hubbell the agreement with the proposed formulation is excellent. This is true although Mendel's animals are quite a bit

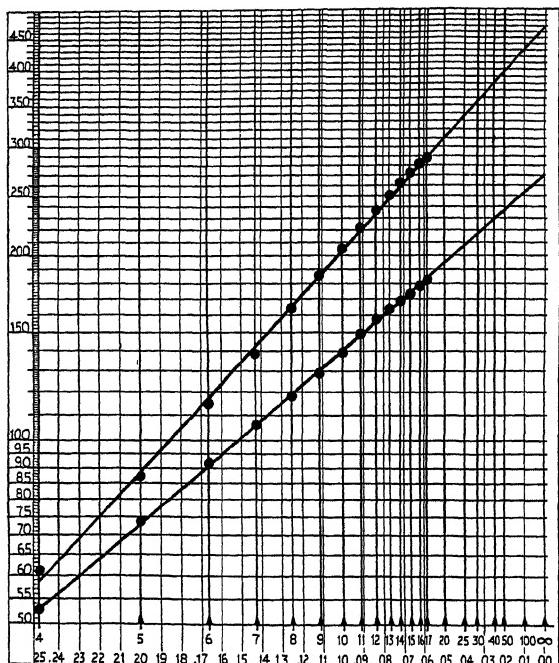


Fig. 3 The lower curve gives the mean values for 144 females reared for breeding purposes in 1937. The upper curve represents the weights of forty-two males reared during 1936 and 1937. The graph paper is that used for routine records. The lines are the best fit to the combined 1936 and 1937 data, obtained by the method of least squares, which calculation also gives directly the values for  $k$  and  $A$ . Fitting can also be done by eye;  $A$  can then be read off the fitted line at infinity on the time scale, as indicated in the figure, and  $k$  can be calculated from any two points on the line, using the formula for  $k$  given in the text.

larger, having been selectively bred for size, as a part of the program of developing large rats, referred to in a number of papers from Mendel's laboratory. The size difference of genetic origin (Mendel's rats and ours are of the same original strain) has no effect on the shape of the growth curve, both

following the log-reciprocal formula equally well. The only difference is in the constant A.

In figure 2 we have illustrated the occurrence of rats of two different inherent average sizes with the same growth k. It is now of interest to ascertain to what extent k and A will vary in other rat colonies. We have found in the literature data on growth of rats which will throw some light on this point. Some of these have been collected in table 2. It will be seen that for albino rats both the male and female k's vary little from their

TABLE 2  
*Constants of log-reciprocal equation.*

	FEMALES			MALES			$k_{male}$
	Age range	k	A	Age range	k	A	$k_{female}$
Authors' data <sup>1, 3</sup>	4-50	2.84	270	4-17	3.65	470	1.29
Mendel-Hubbell ('35), <sup>2</sup> 1935 rats	3-16	2.84	380	3-39	3.73	650	1.31
Smith-Bing ('28)	3-33	2.76	316	3-33	3.67	548	1.33
Macy et al. ('27) — group labelled "stock diet" <sup>2</sup>	4-30	2.86	278	4-30	3.66	484	1.28
Freudenberger ('32) <sup>3</sup> — albinos	3-64	2.80	282	3-64	3.70	440	1.32
King ('15) <sup>1, 3, 4</sup>	4-70	2.98 2.84	251	4-70	3.73	360	1.25 1.31
Sperry-Stoyanoff, Series III ('34) <sup>5</sup>				3-22	3.68	476	

<sup>1</sup> Females known to have been bred regularly and weighed between pregnancies.

<sup>2</sup> The original observations were not available; the points used in fitting were read off the published smoothed curves.

<sup>3</sup> Rat groups a year or more old present a problem with regard to sickness and death, as mentioned below in connection with the Evans data. Freudenberger states that a not negligible number of abnormal weights were included in his mean values. The end of the curve therefore falls off the log-reciprocal line just perceptibly in the males, and quite obviously in the females. King applied a correction in her published figures. We have likewise done so.

<sup>4</sup> The King data are among the earliest. The diet—"selected table scraps"—could, however, very well be good by current standards, and we have included the data because of the generally excellent fit to the equation. The points have a very unfortunate distribution on a log-reciprocal plot because of the large (30 day) interval between weighings. The decision between 2.98 and 2.84 for the female k rests on one point (the 30 day weight). The A value is not affected. We are inclined to accept the figure 2.84, particularly since the male data show no peculiarity.

<sup>5</sup> Detailed growth data kindly supplied by Dr. Sperry.

means. The ratio  $\frac{k_{male}}{k_{female}}$  is constant within about 3% of the mean.

The thought that the formula represents the inherent qualities of the growth curve is further substantiated in the case of animals artificially stimulated beyond their normal growth. Curve 4 in figure 1 and in figure 2 relates to data of Evans ('23-'24) on female rats of the pied Long-Evans strain, injected daily with anterior pituitary substance. In Evans' table of individual weights a certain number of deaths are recorded and a few other cases of definite loss in weight. Since we are discussing normal growth, we have omitted from the mean values all weight data indicative of either definitely abnormal conditions or approaching death. This procedure brought about an improvement in the smoothness of the curve. These animals, similar in weaning weight to the rats of curve 2, outgrow, under the influence of the pituitary substance, not only the animals of curve 2 but also the much larger ones of curve 3. In the log-reciprocal plot, after a short latent period, they also follow a straight line. The uninjected controls follow a course essentially a straight line prolongation of the latent period (fig. 2).

It is apparent therefore that neither size nor growth rate appears to affect that growth property of rats which causes them to increase their weight according to the scheme expressed by our empirical formula, as long as they are on diets such as the present-day good stock diets free from known growth inhibiting deficiencies.

*Individual growth curves.* This remarkable adherence to a simple formulation by so many different colonies, with apparently all differences in growth among albinos of the same sex controlled by a single variable (A), raises a question as to the growth curves of individual rats. The data given so far are all based upon means of large numbers. What is the course of the individual curves? Is the constancy of k due to the averaging of large numbers of different k's with essentially the same distribution in each colony, or is this a feature of the individual curves also?

It has been found that many individual curves show excellent adherence to a log-reciprocal line of slope 2.84. The error on points which make up an individual rat's growth curve is small enough so that it can be fairly said that most of the animals adhere satisfactorily to the formula with this particular slope. Since size (as represented by A) varies over a certain range, this results in a series of parallel lines on log-reciprocal paper. When k, the slope, and log A, the intercept, are calculated by least squares for individual rats, a certain range of k, as well as of A, is observed. These deviations from the mean slope present a real problem, for some deviations in the calculated slopes would be expected (experimental error) even if the true slopes were all identical. The limitations of the least squares method and the difficulty of error calculations on such data become very apparent. We are not able to say that in the case of individual albino rats we have been able to establish the existence of several different k values; the balance of evidence goes to show that the apparent differences are due to error in the original data, and that albino rats of our colony adhere to a single slope with moderate error. An actual range of size (i.e., A value) beyond the experimental error seems to be established. This view on albino rat growth is strengthened by the fact that a strain bred from hybrids (Long-Evans strain) shows in our hands an easy division into individuals of small initial size with large slope, and large initial size with small slope. This is at least in part verified by the study of various data on Long-Evans rats from several other laboratories; the k's for various colonies are all different, and even our own Long-Evans colony k is not quite the same from year to year.<sup>2</sup> It is plausible enough from a

<sup>2</sup>It is interesting to note that the identity of the ratio of male to female k from colony to colony holds for Long-Evans rats as well as albinos. This constancy is hardly accidental, and can probably be taken as corroboration of the thought that the formula gives a useful picture of the course of growth in the rat. It also means that in individual Long-Evans colonies the male k changes pari-passu with the female k. There are enough data on the distribution of coat color patterns in the various experimental records on Long-Evans rats so that on mere inspection it seems evident that one whole colony (males and females) may differ characteristically in its genetic composition from another.

genetic point of view that the hybridization of two very different strains (as in Long-Evans rats) should leave the haphazardly bred colony with at least some representatives of two initially different growth slopes. It is admitted that this may possibly also occur in the albino rats, but if so the differences must be small, since it has not been possible so far to establish proof for such a condition.

During the time these data have been collected we have found a few individual rats which obviously do not follow our growth curve. In some of these food consumption had been recorded and it was found that subnormal growth was accompanied by subnormal food intake. Sooner or later unmistakable signs of pathological state (usually respiratory infection) became evident. On several occasions an unusually high infant mortality has developed in the colony, passing off again without a definite cause being ascertained. At such times the surviving rats have shown individual deviations from the growth curve larger than usual during the early part of the curve with good adherence later on. Just recently we have for the first time (in over 3 years) had an isolated group of ten rats show an unprecedentedly high growth slope from 4 to 8 weeks. This might be due to undernutrition before or during the weaning time with a modified realimentation. We have, however, no concrete explanation to offer. All these exceptions taken together have not enough weight to lessen materially our belief that *normal* rat growth follows the scheme here outlined, but have rather stimulated our interest in detecting and eliminating abnormalities.

*Spontaneous realimentation.* We have cited a number of instances where informed efforts to eliminate deficiencies have led to post-weaning growth curves which satisfy the conditions of the proposed formula even though the intrinsic size of the animal varies. Deviation from the formula with experimentally varied deficiencies will be dealt with in future reports. However, there are in the literature numerous examples (Donaldson and others) where growth according to the formula has not been achieved and it seems likely that the reason

for this deviation is to be found in the existence of dietary deficiencies. The Donaldson curve has already been presented in figure 1 and figure 2. These data were obtained at a time when little was known concerning nutritional requirements. They illustrate a common type (though not the only one) of deviation from formula growth. On linear paper the curve is sigmoid, i.e., a slow initial rate of growth is followed by a larger rate after which again the rate decreases. The same features are illustrated in many of the early Mendel rats and the Greenman and Duhring data (Donaldson, '24). The diets employed allowed the animals to grow and propagate, and so were satisfactory as stock diets. They were, however, distinctly inferior to the type of diet most investigators now choose, in line with the thought expressed by Freudenberg ('33) that ". . . it is well known that the rat thrives much better on our more modern diets." The particular peculiarity of these curves is that the rate of growth increases temporarily after weaning; while on the newer (better) diets the rate falls off continuously from 4 weeks on. Judging by growth rate the Donaldson animals are in a better nutritional condition after about the tenth week than from the third to the seventh week. This improvement with no change in diet resembles the finding of high growth rates shown by Osborne and Mendel ('16), Clark and Smith ('38), Jackson ('36, '37) and others to be characteristic of the realimentation or recovery curve obtained when rats are changed from a poorer to a better diet. Similarly in the log-reciprocal plotting (fig. 2) the final  $k$  value of Donaldson is "normal" but straight lines fitted to intermediate portions of the curve would indicate unusually high  $k$  values (recovery), while the initial portion of the curve has a very low slope. The "normal" final  $k$  value together with the intermediate high linear rate or high  $k$  values as against the low initial rate or  $k$  indicates that actually the diet at later ages satisfies the growth requirement better than at an early age. This is not inconsistent with the many findings to the effect that signs of deficiency are more easily produced at early ages or that requirements in the young are particularly

critical. We give this explanation for the sigmoid feature of deficient growth very tentatively. We give it at all only because it appears that the subject matter is of considerable interest. We have in mind particularly the many references to "preadolescent growth spurt" etc., as a normal or unavoidable phenomenon in growth.

With moderate or mild deficiencies a post-weaning curve may result which in linear plotting is only doubtfully sigmoid or not at all so and still does not fit the proposed formula. Diet

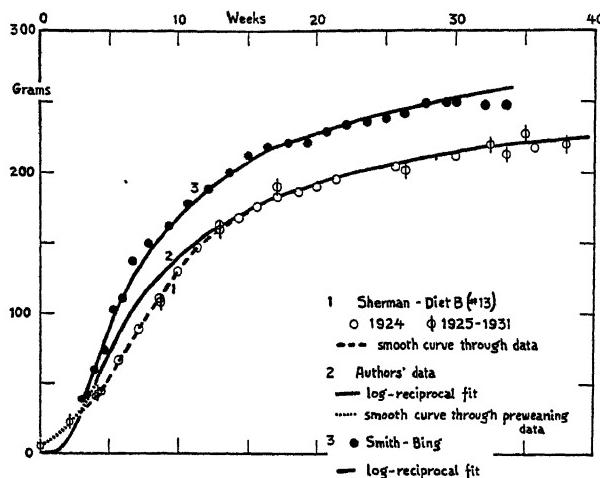


Fig. 4 See text.

B (no. 13) of Sherman is considered a normal diet and is used for studying the effect of supplements. Figure 4, curve 1, shows the growth on Sherman's diet B ('24, '25, '26, '31) compared with that on our stock diet (curve 2) and that of Smith and Bing ('28), already mentioned in table 2. These animals are all females of the same original strain; our colony is an offshoot of Sherman's, and his in turn an offshoot of the Osborne-Mendel colony, which is represented by the rats of Smith and Bing. Smith and Bing's rats were selectively bred for size; Sherman's and ours were not, as is demonstrated by the identical final size. The difference between diet B and that

of Smith and Bing is that the latter has a lettuce supplement, substitutes calcium carbonate for some of the sodium chloride, and gives nursing mothers a supplement of yeast. A comparison of the latter two curves shows clearly that growth according to our equation can be achieved by judiciously supplementing a deficient diet. Rats raised for generations upon Sherman's diet B and on our stock diet apparently show no significant differences in weight very early in life and as adults. The deficit during the period from 4 to 13 weeks is entirely in line with what has been said above concerning the apparent spontaneous realimentation on quite inadequate diets.

*Pre-weaning growth.* We have emphasized that the proposed formulation applies from the 4 weeks weaning time onward as far as suitable data are available. This is the phase of growth, auxano-differentiation of Huxley (Huxley, '32, p. 148) in which the weight of the animal increases essentially on the basis of completed development of its parts. The earlier phase (conception to weaning) involves largely the differentiation of the various organs and organ systems (Huxley's histo-differentiation). The approximate location of the transition from histo- to auxano-differentiation in the rat at 3 to 4 weeks rests on a variety of evidence (Freudenberger, '32: definite change in proportion of measurements ceases at about 3 weeks; Donaldson, '24: fully functional state of sense organs sets in at this time; Jackson, '32: extended discussion; Cole, '37: successful hormonal stimulation of sex organs in female is possible any time after 3 weeks but not before). We would therefore, both a priori and on the basis of Huxley's discussion, not expect a given simple expression to fit both phases of growth. The log reciprocal formula includes a point of inflection at about 3 weeks, and the curve turns in the right direction. In harmony with what has been said concerning histo- and auxano-differentiation it does not, however, give even an approximation to the data from birth to 3 weeks, as can be seen graphically in figure 4. All this has no bearing on the practical objectives as outlined in this report, but involves

a number of interesting problems along the lines laid down by Huxley.

In our experience the weight at 3 weeks often falls on the log reciprocal graph but at other times even the 4-week weight gives not as good a fit as later on. In many respects it is unfortunate that animals are usually selected for assignment to experimental groups at an age associated with the marked transition character described above as well as with the dietary changes consequent to weaning.

*Significance of the proposed formulation.* In this discussion we are not interested in the question raised by Osborne and Mendel ('26) years ago whether maximal growth is most advantageous to the animals, etc., nor are we attempting to define "normal" growth. The basic thought we are developing in this and several succeeding reports is that on diets adequate for best growth, i.e., diets in which no experimental restriction is placed on the natural optimal growth inherent in the species, we find that relations of weight to time, weight to food intake and to relative weights of parts of the body, etc., are simpler in form than on deficient diets where natural growth is inhibited. If effects of deficiencies are to be elucidated an unhampered norm should first be worked out. We merely point out that removal of all known deficiencies which arbitrarily hamper growth would be expected to lead to simpler conditions as far as growth is concerned, and does in fact produce a simpler scheme of weight increase.

While we are not attempting any rationalization of the growth formula here proposed, it may not be out of place to call attention to the fact that it is in line with two well-known concepts regarding growth which recur constantly in the literature on the general biology of growth. These are very succinctly formulated by Huxley ('32, p. 6). "One essential fact about growth is that it is a process of self-multiplication of living substance, i.e., that the rate of growth of an organism growing equally in all its parts is at any moment proportional to the size of the organism. The second fundamental fact about growth is that the rate of self-multiplication slows down with

increasing age (size)." The first of these concepts is expressed in our formulation by logarithmic plotting of the weight, the second by the reciprocal time axis.

#### SUMMARY

1. The growth of rats on present-day good stock diets is characterized by a progressive decrease in gain for each successive time interval after weaning.
2. Such data can be conveniently plotted as a straight line if the logarithm of the weight and the reciprocal of time are used as coordinates. The formula is:

$$\log W = \frac{-k}{t} + \log A.$$

These findings are corroborated by numerous data of other investigators.

3. Neither natural variation in size of the animal nor artificial stimulation of growth rate causes a deviation from the empirical formula of growth. While inherent size of rats varies considerably, the slope of the plot ( $k$  of the formula) varies but little for each sex in albino rats. In all cases where data for males and females are available, the ratio of  $k$  for males and  $k$  for females is constant within 3%.

4. Deviations on diets suboptimal for growth are discussed.

#### LITERATURE CITED

- CLARK, M. F., AND A. H. SMITH 1938 Recovery following suppression of growth in the rat. *J. Nutrition*, vol. 15, p. 245.
- COLE, H. H. 1937 Superfecundity in rats treated with mare gonadotropic hormone. *Am. J. Physiol.*, vol. 119, p. 704.
- DAWSON, A. B. 1925 The age order of epiphyseal union in the long bones of the albino rat. *Anat. Rec.*, vol. 31, p. 1.
- DONALDSON, H. H. 1924 The Rat. *Memoirs of The Wistar Institute of Anatomy and Biology*, No. 6.
- DONALDSON, H. H., E. H. DUNN AND J. B. WATSON 1906 A comparison of the white rat with man in respect to the growth of the entire body. *Boas Anniversary Volume*, pp. 5-26. G. E. Stechert & Co., New York.
- EVANS, H. M. 1923-1924 The function of the anterior hypophysis. *Harvey Lectures*.

- FREUDENBERGER, C. B. 1932 A comparison of the Wistar albino and the Long-Evans hybrid strain of the Norway rat. *Am. J. Anat.*, vol. 50, p. 293.
- \_\_\_\_\_. 1933 Variability in body length, body weight, and organ weights of the rat. *Anat. Rec.*, vol. 56, p. 47.
- HUXLEY, J. S. 1932 Problems of relative growth. Methuen and Co., London.
- JACKSON, C. M. 1932 Structural changes when growth is suppressed by under-nourishment in the albino rat. *Am. J. Anat.*, vol. 51, p. 347.
- \_\_\_\_\_. 1936 Recovery in rats upon refeeding after prolonged suppression of growth by dietary deficiency of protein. *Am. J. Anat.*, vol. 58, p. 179.
- \_\_\_\_\_. 1937 Recovery of rats upon refeeding after prolonged suppression of growth by underfeeding. *Anat. Rec.*, vol. 68, p. 371.
- KING, H. D. 1915 The growth and variability in the body weight of the albino rat. *Anat. Rec.*, vol. 9, p. 751.
- MACY, I. G., J. OUTHOUSE, M. L. LONG AND A. GRAHAM 1927 Human milk studies. I. Technique employed in vitamin studies. *J. Biol. Chem.*, vol. 73, p. 153.
- MCCAY, C. M., L. A. MAYNARD, G. SPERLING AND L. L. BARNES 1939 Retarded growth, life span, ultimate body size and age changes in the albino rat after feeding diets restricted in calories. *J. Nutrition*, vol. 18, p. 1.
- MENDEL, L. B., AND R. B. HUBBELL 1935 The relation of the rate of growth to diet. III. A comparison of stock rations used in the breeding colony at the Conn. Agr. Exp. Station. *J. Nutrition*, vol. 10, p. 557.
- OSBORNE, T. B., AND L. B. MENDEL 1916 Acceleration of growth after retardation. *Am. J. Physiol.*, vol. 40, p. 16.
- \_\_\_\_\_. 1926 The relation of growth to diet. I. *J. Biol. Chem.*, vol. 69, p. 661.
- SHERMAN, H. C., AND H. L. CAMPBELL 1924 Growth and reproduction upon simplified food supply. IV. Improvement in nutrition resulting from an increased proportion of milk in the diet. *J. Biol. Chem.*, vol. 60, p. 5.
- \_\_\_\_\_. 1934 Observations on growth from the viewpoint of statistical interpretation. *Proc. Nat. Acad. Sci.*, vol. 20, p. 413.
- SHERMAN, H. C., AND F. L. MACLEOD 1925 The calcium content of the body in relation to age, growth and food. *J. Biol. Chem.*, vol. 64, p. 429.
- SHERMAN, H. C., AND E. J. QUINN 1926 The phosphorus content of the body in relation to age, growth and food. *J. Biol. Chem.*, vol. 67, p. 667.
- SHERMAN, H. C., AND L. E. BOOHER 1931 The calcium content of the body in relation to that of the food. *J. Biol. Chem.*, vol. 93, p. 93.
- SMITH, A. H., AND F. C. BING 1928 Improved rate of growth of stock albino rats. *J. Nutrition*, vol. 1, p. 179.
- SPERRY, W. M., AND V. A. STOYANOFF 1934 Effects of long-continued cholesterol feeding in rats. *J. Nutrition*, vol. 9, p. 131.

# GROWTH AND CALCIFICATION ON A DIET DEFICIENT IN PHOSPHATE BUT OTHERWISE ADEQUATE

THEODORE F. ZUCKER, LILIAN HALL AND MARGARET YOUNG

*Department of Pathology, College of Physicians and Surgeons,  
Columbia University, New York*

## FOUR FIGURES

(Received for publication January 28, 1941)

The finding that experimental rickets can be produced in rats came not by systematic design of diets for this purpose, but through the examination of bones of animals which had been kept on diets developed for other much more general purposes. Of the two pioneer diets no. 84 of Sherman and Pappenheimer ('21) was simple in its makeup but had marked multiple deficiencies resulting in very little growth and was uncertain in its action, while no. 3143 of McCollum et al. ('21) gave more reproducible results and fair growth but was quite complicated. A simplifying step was made when Steenbock and Black ('25) published their diet no. 2965 which was easy to compound, satisfactory as to reliability and also gave better growth than no. 84. For purpose of bringing about rickets for ordinary curative experiments it has stood the test of time and is justifiably more generally used than any of the other available diets. For certain purposes, however, qualities not shared by any of the diets mentioned are desirable. This is attested to by Jones ('38), by Schneider and Steenbock ('39), and by others.

A number of diets deficient only in phosphate have been devised, most of them containing less phosphorus and producing a more marked rickets than the traditional rachitogenic

diets. The present report deals with a diet deficient only in phosphate (no. 803), which, however, produces rickets to about the same extent as no. 2965. This allows a direct comparison of the two diets, the intensity of the rickets production and the calcium content being the same. The similarity in rickets producing quality is probably the resultant of a higher available phosphorus content in no. 803 accompanied by better growth. In the ration no. R14 of Schneider and Steenbock ('39), which with phosphate supplements produces excellent growth, the phosphorus is very low and calcium also proportionately lower. The interesting and rather unexpected results recorded by these authors show that comparison with no. 2965 is quite a bit more complicated.

#### EXPERIMENTAL

Rats from our albino colony were weaned at 4 weeks of age and placed upon various rations: no. 2965, our diet no. 803 and our stock diet (see preceding paper, Zucker et al., '41, for composition); no. 2965 and no. 803 supplemented with 2.0%  $\text{KH}_2\text{PO}_4$  (an amount shown to give optimal results); all three diets supplemented with adequate vitamin D; no. 803 supplemented with both phosphate and D.

The composition of diet no. 803 is, in per cent: starch, 65; cottonseed flour, 20; casein, 6; egg albumen, 3; NaCl, 1; cottonseed oil containing carotene, 2;  $\text{CaCO}_3$ , 3. The diet contains 0.28% of phosphorus, of which 52% is in the form of phytin. The phosphorus content is about the same as average values for no. 2965, but the phytin fraction is lower than is found in analyses of most samples of no. 2965 according to Harris and Bunker ('35). The carotene being mixed into the diet was put at a probably unnecessarily high level equivalent to 750 units of A per 100 gm. of diet. The cottonseed flour contains 40% of a relatively good quality of protein and also satisfies the B complex requirements.

On account of seasonal, or recurrent aperiodic variations, it is always desirable to conduct all experiments to be compared with each other at the same time. This was obviously

not possible in this extended series. In general, however, it can be said that since a large part of the data covers a planned distribution and no marked seasonal variations happened to enter, the results are sufficiently comparable. In two cases where seasonal variation had to be carefully excluded special seasonal comparisons were made (see table 2 and fig. 3).

TABLE 1

*Body weight from fourth to seventh week and per cent bone ash at 7 weeks of age.*

DIET	SEX	N	WEIGHTS				PER CENT BONE ASH AT 7 WKS.	GROUP NO.
			Age 4 wks. gm.	5 wks. gm.	6 wks. gm.	7 wks. gm.		
Stock	F	112	45.4±3.8	60.3	75.0	86.9±12.7	58.0±1.4	2
	M	112	49.1±5.7	66.6	84.9	101.1±17.8	57.6±1.5	
Stock + D	F	51	45.4±4.0	61.2	78.1	90.8±15.0	57.9±1.2	1
	M	49	50.6±5.9	68.6	89.0	104.3±20.7	57.3±1.6	
803	F	241	45.4±6.2	52.5	62.3	71.0±10.2	39.3±3.7	5
	M	253	47.8±6.3	56.8	65.1	76.8±11.9	37.0±3.6	
803 + D	F	43	43.8±4.1	49.6	58.4	68.6±7.8	51.6±1.4	6
	M	49	46.2±5.7	52.2	62.9	72.7±12.8	50.2±1.8	
803 + P	F	39	43.6±2.6	58.8	72.6	83.5±9.3	58.0±1.0	4
	M	49	46.9±4.8	65.0	81.0	97.4±14.7	56.9±1.3	
803 + P + D	F	25	43.4±3.1	59.7	73.6	85.6±9.6	59.3±1.0	3
	M	25	47.2±4.8	65.9	80.8	94.5±15.8	58.5±1.1	
2965	F	40	43.3±3.3	49.2	55.5	60.8±8.8	37.4±3.9	8
	M	56	44.6±5.5	50.3	56.8	63.4±9.7	36.1±4.6	
2965 + D	F	26	43.4±2.8	48.2	54.5	60.4±5.0	49.8±1.6	9
	M	40	46.0±4.6	51.4	58.8	64.7±8.5	49.1±1.8	
2965 + P	F	13	43.6±2.2	50.8	57.6	65.6±9.6	55.7±1.6	7
	M	7	48.0±3.6	55.6	61.7	67.3±10.6	53.4±2.3	

The  $\pm$  value given after the mean is the standard deviation,  $\sigma = \sqrt{\frac{\sum d^2}{N}}$ . Group numbers are the identifying numbers used in figures 1 and 2. N = number of animals.

Weekly weighings were made over the 3 week experimental period. At 7 weeks the rats were autopsied. Two criteria of the extent of rickets were used: the percentage of ash in the dry fat-free femur, and the width of the uncalcified cartilage (rachitic metaphysis) of the tibia. The latter has been less extensively employed but if properly measured is of equal significance (Steenbock et al., '25; Dodds and Cameron, '38;

Russell et al., '39). The cartilage width measurements were made on enlarged photographs ( $\times 3.3$ ) of split silver-stained tibias, and as reported here are 3.3 times natural width. Further details of the technique, as well as a detailed comparison with ash percentages at various levels of rickets production will have to be reserved for another report. The mean values are presented in tables 1 and 2.

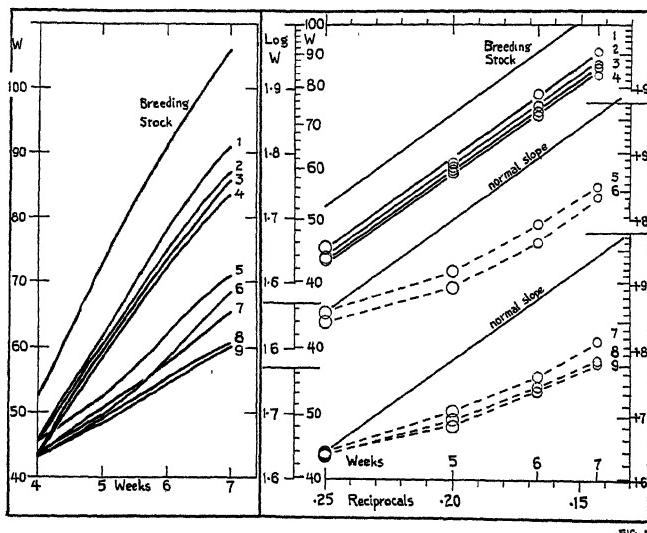


FIG 1

Fig. 1—left hand section Growth of females on the nine rations, plotted as weight against time. 1—stock + D; 2—stock; 3—no. 803 + P + D; no. 803 + P; 5—no. 803; 6—no. 803 + D; 7—no. 2965 + P; 8—no. 2965; 9—no. 2965 + D.

Fig. 1—right-hand section The data of left-hand section replotted as the logarithm of the weight against the reciprocal of time. Each mean weight is represented by a circle of radius 1 gm. In order to avoid crowding the curves have been separated into three groups, each one plotted on a different weight scale (i.e., different origin).

#### DISCUSSION

**Growth.** Figure 1 shows the growth of the females on the various rations. The left-hand section of figure 1 shows the data as plotted on ordinary coordinate paper while the right-hand part of the figure makes use of the log-reciprocal plotting

as described in the preceding paper. It can readily be seen that the addition of vitamin D in amounts sufficient to prevent rickets on either rachitogenic diet does not significantly alter the growth during 3 weeks<sup>1</sup> (compare 5 with 6, and 8 with 9, in fig. 1). The phosphate supplement added to no. 2965 (group 7) enhances the growth somewhat and of course prevents rickets. The diet thus supplemented to make it non-rachitogenic, however, does not result in growth as good as that obtained with rickets producing diet no. 803 (group 5). With no. 803 a suitable phosphate supplement (group 4) carries the growth into the range of that recorded for the two stock diet experiments (with D: group 1; without D: group 2).

For a further analysis of these results it is necessary to turn to the log-reciprocal graphs (fig. 1, right-hand section). The growth of rats on diets with no known deficiencies shows a straight line of constant slope when the logarithm of the body weight is plotted against the reciprocal of time (Zucker et al., '41). All deficiencies tried so far result in deviations from this line, which deviations will vary according to the type and degree of the deficiency. The growths on no. 2965 (group 8), no. 2965 plus D (group 9), no. 2965 plus phosphate (group 7), no. 803 (group 5), and no. 803 plus D (group 6) are obviously characteristically deficient. Diets no. 803 plus phosphate (group 4), no. 803 plus phosphate plus D (group 3), and the stock diet (groups 2, 1) each plot quite acceptable straight lines with slopes differing to an insignificant extent from our colony norm. It will be noted that this formulation allows one to compare quality of growth in groups of different inherent size. Under these conditions neither body weight achieved nor gain in weight is a suitable measure.

It can be reasonably concluded that diet no. 803 is deficient only in phosphate, since the addition of adequate phosphate

<sup>1</sup> A well-known fact for diets of the low phosphorus rickets producing type. This must be carefully distinguished from the marked effect on growth of the addition of vitamin D to diets in which there is a relative calcium deficiency. See Zucker, Hall and Young ('38), and Krieger, Bunkfeldt and Steenbock ('40).

(group 4) results in normal growth<sup>2</sup> with rather exacting criteria applied to the term "normal". A further vitamin D supplement (group 3) produces no change in growth just as is the case when it is added to the stock diet.

*The intensity of rickets produced on diet no. 803 as compared with diet no. 2965.* Table 2 presents data on bone ash per cent and cartilage width measurements obtained from comparable experiments on the two diets. In order to avoid the possible criticism that seasonal variation might play a role we have selected from table 1 rats from experiments which

TABLE 2  
*Comparison of rickets production on two rachitogenic diets.*

DIET	NO. OF ANIMALS		CARTILAGE WIDTH		BONE ASH %	
	♀	♂	♀	♂	♀	♂
21 day results						
2965	21	26	5.75±0.18	5.42±0.29	39.06±0.92	37.86±1.11
803	32	36	5.70±0.14	6.26±0.17	38.61±0.75	36.13±0.70
31 day results						
2965	17	20	6.73±0.15	6.95±0.13	35.26±1.03	35.07±0.70
803	16	17	6.63±0.31	6.70±0.32	36.11±1.63	33.51±0.94

The ± figure after the mean value is the standard error  $\sigma_m = \frac{\sigma}{\sqrt{N}}$

were conducted at the same time. These animals as indicated above were on the diet for 21 days, and are given in the first section of table 2. The second section deals with similarly treated rats, but the length of the experimental period was 31 days. The difference between the effects of the two diets cannot be considered significant. We have then two diets of the same rickets producing quality as expressed by two separate criteria. There is, however, a difference of some importance; the rickets on no. 803 is uncomplicated rickets since the

<sup>2</sup> While the 3 weeks following weaning cover the most critical period (see Zucker et al., '41), this short time interval hardly constitutes rigorous evidence. Unpublished results have however established that the data obtained with various suitable levels of calcium and phosphorus as supplements to the basal portion of diet no. 803 adhere to the formula for 11 weeks. Incidentally it may be noted that the body weights given by Schneider and Steenbock ('39) on diet no. R14 supplemented with phosphate also give a very acceptable straight line in log-reciprocal plotting.

addition of the single deficient factor (phosphate) produces normal animals both in respect to bone ash per cent and growth. In the case of diet no. 2965 there are other complicating deficiencies besides that of phosphate.

*The effects of phosphate and of vitamin D when added to the rickets producing diets.* As mentioned above the supplements of phosphate and of vitamin D produced maximal effects as far as rickets prevention is concerned. Larger doses of phosphate tended to the production of low calcium rickets. Further increase in D produced no greater an effect on bone ash or bone picture than that described for the 2 units per day. In each case the cartilage width of the tibias was about 1 mm., which is the value found in stock diet animals of the same age (7 weeks). The 1 mm. in the enlarged photograph is 3.3 times the actual width, and therefore in excellent agreement with the  $350\ \mu$  actual width recorded by Dodds and Cameron ('38). Microscopic sections also revealed nothing to distinguish such bones from the normal. It is therefore apparent that the supplements here given either to rats on no. 2965 or on no. 803 yielded a bone which was anatomically normal.

In sharp contrast with this uniformity of effect is the widely varying percentage of bone ash (group means 49% to 59%), as well as the considerable range of body weight (group means 60 gm. to 85 gm.).

It is known (see, e.g., Dutcher et al., '25) that the ash per cent in bone increases steadily with age (weight). This is also apparent from the data of Sherman et al. ('25, '31) on per cent of calcium in the body of the rat. Certain generalizations have been made with regard to the relation of parts or components of the body to the body as a whole during the course of growth. Thus Needham ('34) has shown that, using the data of Sherman, the logarithm of the calcium content plots a straight line against the logarithm of the body weight. In view of Needham's reasoning, as well as many other recorded facts, it is advisable to consider body weight in any discussion of the meaning of percentage of ash in the bone.

In figure 2 we have plotted as small points the logarithm of the per cent of bone ash against the logarithm of the body weight of 346 stock diet females varying in body weight from 30 to 150 gm. This gives a picture of the change in bone ash per cent on body weight as well as of the variability encountered in our colony. The heavy-filled circles are the data given in table 1 for females. The lengths of the dotted lines through the points represent the standard deviations on both axes ( $\sigma = \sqrt{\frac{\sum d^2}{N}}$ ). The identifying numbers for the points are the same as those used for the curves in figure 1 (in order of

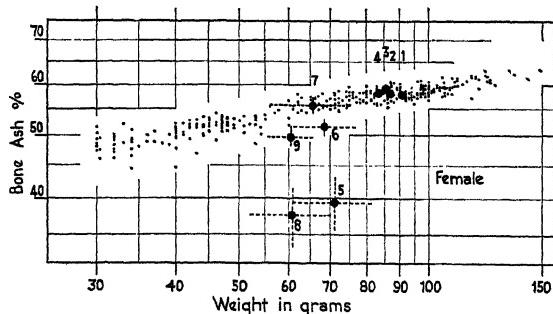


Fig. 2 Log-log plot of bone ash per cent against body weight for females. The small dots represent individual normal stock females, 346 in all, with an age range of 24 to 77 days. It is apparent that this kind of plotting results in a straight line. The large solid circles are the means of table 1 for the females, numbered as before: 1—stock + D; 2—stock; 3—no. 803 + P + D; 4—no. 803 + P; 5—no. 803; 6—no. 803 + D; 7—no. 2965 + P; 8—no. 2965; 9—no. 2965 + D. The lines running through these points represent the standard deviations of the bone ash per cent and the weight. Supplementing either rachitic diet with phosphate results in normal bone ash for the weight achieved; supplementing with D results in bone ashes significantly low even for the weight achieved.

descending final weight). The two stock diet groups (1, 2) as well as the phosphate supplemented groups on no. 803 (3, 4), fall within the range of the small points at body weights normal for rats of the age given (see fig. 1-B). The points for the two unsupplemented rachitogenic diets (5 = no. 803; 8 = no. 2965) fall far below the normal range even for their low body weights. The phosphate supplemented diet no. 2965 (group 7) produces a bone ash percentage not normal for its age, but

precisely normal for the body weight attained. The two remaining points ( $6 = \text{no. } 803 + D$ ;  $9 = \text{no. } 2965 + D$ ) are the ones of particular interest. The question is: does a vitamin D supplement produce bone ash normal either for age or for body weight? The answer is apparently negative, since they fall with the small standard deviation indicated outside the range of ash for normal stock diet animals. Here again the matter of seasonal effects arises. In figure 3 we show those individual values represented in the mean value of point 6 (no.  $803 + D$ ) in figure 2 which could be seasonally balanced with stock diet animals, together with the corresponding males.

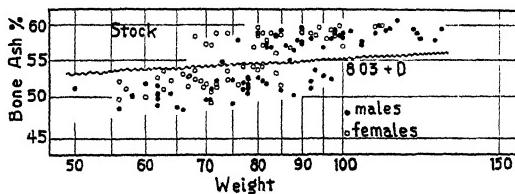


Fig. 3 Log—log plot of bone ash per cent against body weight for males and females. Below the waved line are individual points from the group on no.  $803 + D$  obtained over a short period of time; above the waved line are all the available data on 7-week-old normals on the stock diet covering the same time.

These are all the data points below the waved line. For comparison we have plotted data (male and female) obtained on stock diet animals of the same age, which covered the calendar time of the data on no.  $803 + D$ . Since there is not even any overlapping of individual values, the conclusion seems warranted that diet no.  $803 + 2$  units of D daily does not result in normal bone ash percentages either for age or for body weight. The same conclusion applies to no.  $2965 + D$  (point 9 in fig. 2). On whatever basis the comparison is made it is apparent that under these conditions—i.e., the usual type of low phosphorus rickets production—optimal doses of vitamin D produce less effect than optimal phosphate supplements.

It is usually stated that vitamin D prevents the rickets of rats on the low phosphorus type of rachitogenic diet. This apparently is not true unless one wants to define rickets on

exclusively morphological grounds. Indications of this were published as early as 1925 (Dutcher et al.). The observation that vitamin D cannot fully replace an absolute phosphate deficiency is hardly worth a lengthy discussion. The interest lies in the fact that some important differences between the usual type of experimental rickets in rats on the one hand, and the spontaneous rickets commonly found in chicks and infants on the other hand, are set out clearly by the conditions here described. In chicks bone ash per cent is brought to normal when under usual conditions vitamin D action prevents the anatomical signs of rickets. In infants calcium and phosphorus retention, and therefore presumably the composition of the bone, are normal under conditions of optimal vitamin D action, while without other changes in the diet, inadequate vitamin D results in rickets. It may, however, even with chicks and infants be quite fallacious to assume that calcium and phosphorus deposition must under all conditions be paralleled by the morphological appearance of the bone, or specifically that normal morphology means normal bone composition. Under the usual conditions of observation infantile rickets and chick rickets can be considered a vitamin D deficiency in the presence of adequate calcium and phosphorus, and the administration of D removes all signs of rickets. Experimental rat rickets is essentially a phosphate deficiency. Only meager attention has been given to the little understood fact that under such conditions vitamin D administration leads to an apparently complete suppression of rachitic bone changes judged visually while in composition the same bone is still quite subnormal, i.e., "rachitic" in the sense of not having one of the chemical criteria of rickets brought into the range of normal. The other chief chemical criterion of rickets, the inorganic blood phosphate, behaves according to Dutcher et al. ('25) in quite the identical manner.

A glance at the values for standard deviation of bone ash reveals a striking difference in variability of normal or near

normal values as against the rachitic ones (see also figs. 2 and 4). This has been previously indicated (Zucker, '36) and is in line with the findings of Massengale and Bills ('36) on chickens. The decrease in variability with very low bone ash values which Bills records does not appear in the rat data here given, possibly because they do not reach the low limiting ash percentage value which, as Bills very logically points out, might again decrease the variability. The statement which we

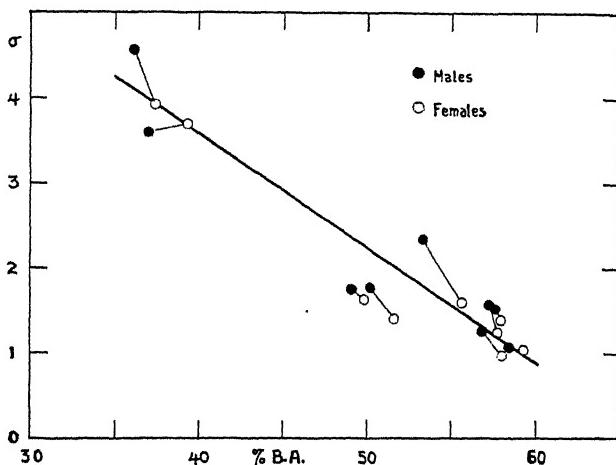


Fig. 4 The standard deviations of the bone ash per cents plotted against the bone ash per cents. The male and female points for each diet are connected. It will be seen that with one exception the male point is to the left of the female point and above it, indicating greater variability and lower bone ash for the males.

(Zucker, '36) have previously made: "It is not surprising to find that the variation in the bone ash values increases the further they are removed from the normal" may have to be amended to take care of the effect of limiting bone ash per cent values. At any rate the data on chicks and on rats are in agreement with respect to the very much larger variability of bone ash per cent values in the rachitic as against the normal range. In quantitative work, as Bills points out, this fact must not be neglected.

## SUMMARY

1. A diet comparable in rickets producing quality to the current rachitogenic diets is shown to be adequate for normal growth when phosphate is added.
2. Addition of vitamin D to this or to other low phosphorus rickets producing diets does not lead to normal growth.
3. Addition of vitamin D to rickets producing diets (low phosphorus rickets) prevents the anatomical signs of rickets but does not produce bone ash per cent values equal to those of stock diet animals of the same age or of the same weight; phosphate addition is required to bring the composition to normal.
4. It is pointed out that the visual signs of rickets do not necessarily correspond to bone composition, and the differences between the experimental rickets of rats and the spontaneous rickets in other species are discussed.

## LITERATURE CITED

- DODDS, G. S., AND H. C. CAMERON 1938 Studies on experimental rickets in rats. II. The healing process in the head of the tibia and other bones. *Am. J. Path.*, vol. 14, p. 273.
- DUTCHER, R. A., M. CREIGHTON AND H. A. ROTHROCK 1925 Vitamin studies. XI. Inorganic blood phosphorus and bone ash in rats fed on normal, rachitic and irradiated diets. *J. Biol. Chem.*, vol. 66, p. 401.
- HARRIS, R. S., AND J. W. BUNKER 1935 The phosphorus of the corn component of the rachitogenic diet. *J. Nutrition*, vol. 9, p. 301.
- JONES, J. H. 1938 The use of fibrin in synthetic diets. *J. Nutrition*, vol. 15, p. 269.
- KRIEGER, C. H., R. BUNKFELDT AND H. STEENBOCK 1940 Cereals and rickets. X. The availability of phytic acid phosphorus. *J. Nutrition*, vol. 20, p. 7.
- MASSENGALE, O. N., AND C. E. BILLS 1936 A quantitative method for the assay of vitamin D with chickens. *J. Nutrition*, vol. 12, p. 429.
- MCCOLLUM, E. V., N. SIMMONDS, P. G. SHIPLEY AND E. A. PARK 1921 Studies on experimental rickets. VIII. The production of rickets by diets low in phosphorus and fat-soluble A. *J. Biol. Chem.*, vol. 47, p. 507.
- NEEDHAM, JOSEPH 1934 Chemical heterogeneity and the ground-plan of animal growth. *Biol. Rev.*, vol. 9, p. 79.
- RUSSELL, W. C., M. W. TAYLOR AND M. T. DUNCAN 1939 Bone development in normal and rachitic rats. *J. Nutrition*, vol. 18, p. 27.
- SCHNEIDER, H. H., AND H. STEENBOCK 1939 A low phosphorus diet and the response of rats to vitamin D. *J. Biol. Chem.*, vol. 128, p. 159.

- SHERMAN, H. C., AND A. M. PAPPENHEIMER 1921 Experimental rickets in rats. I. A diet producing rickets in white rats, and its prevention by the addition of an inorganic salt. *J. Exp. Med.*, vol. 34, p. 189.
- SHERMAN, H. C., AND F. L. MACLEOD 1925 The calcium content of the body in relation to age, growth and food. *J. Biol. Chem.*, vol. 64, p. 429.
- SHERMAN, H. C., AND L. E. BOONER 1931 The calcium content of the body in relation to that of the food. *J. Biol. Chem.*, vol. 93, p. 93.
- STEENBOCK, H., AND A. BLACK 1925 Fat-soluble vitamins. XXIII. The induction of growth promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. *J. Biol. Chem.*, vol. 64, p. 263.
- STEENBOCK, H., E. B. HART, C. A. ELVEHIJEM AND S. W. F. KLETZIEN 1925 Dietary factors influencing calcium assimilation. VI. The antirachitic properties of hays as related to climatic conditions with some observations on the effect of irradiation with ultra-violet light. *J. Biol. Chem.*, vol. 66, p. 425.
- ZUCKER, T. F. 1936 Bone ash in prevention and healing of experimental rat rickets. *Science*, vol. 84, p. 162.
- ZUCKER, T. F., L. HALL AND M. YOUNG 1938 Growth promoting effect of vitamin D on low calcium diets. *Abstracts, 96th meeting Am. Chem. Soc., Div. of Biol. Chem.*, p. 17.
- ZUCKER, T. F., L. HALL, M. YOUNG AND L. ZUCKER 1941 The growth curve of the albino rat in relation to diet. *J. Nutrition*, vol. 22, p. 123.



# THE EFFECT OF CERTAIN FATS AND UNSATURATED FATTY ACIDS UPON THE UTILIZATION OF CAROTENE<sup>1</sup>

W. C. SHERMAN

*Laboratory of Animal Nutrition, Alabama Agricultural Experiment Station, Auburn*

TWO FIGURES

(Received for publication January 7, 1941)

Variations in the growth response of rats receiving carotene or vitamin A in different solvents were observed in the early days of vitamin A research. Subsequent investigations indicated that many of the variations in biological response were attributable to differences in the stability of carotene or vitamin A in various natural fats and synthetic esters.

More recently, however, variations in growth response have been reported to occur even though precautions were taken to insure a uniform intake of the active agent. Lathbury and Greenwood ('34) obtained different biological values for carotene when dissolved in arachis oil and coconut oil and in different samples of these solvents. Kraybill and Shrewsbury ('36) found that two to three times as much carotene was required to give equal growth when fed in decolorized butterfat as when fed in cottonseed oil. Basu ('37) testing several oils, obtained the best growth when carotene or vitamin A was fed in linseed oil and the poorest in arachis oil. De ('37) reported that carotene in foods such as amaranth was not as well utilized as carotene in red palm oil.

<sup>1</sup>A preliminary report of this work was presented before the 34th meeting of the American Society of Biological Chemists at New Orleans, La. J. Biol. Chem., vol. 133, p. lxxxix (1940). Published with the permission of the director of the Alabama Agricultural Experiment Station.

In spite of many reports of variations in growth response with various fats it is not possible to correlate the growth effect with any of their known physical or chemical properties. Moreover, there is no evidence of a direct physiological relationship between the metabolism of vitamin A and any other fat constituent.

Although the composition of the basal diets employed by some investigators has not been published, it appears that a relatively low fat content is essential to demonstrate the greatest effect on the growth response to the various oils. However, the possibility of a growth response from the unsaturated fatty acids supplied by certain oils to vitamin A-free diets apparently has not been given due consideration. Burr et al. ('29, '32) demonstrated the essential nature of unsaturated fatty acids and reported that rats receiving a low-fat diet ceased growing soon after the appearance of the early outward signs of an unhealthy condition (seventieth to ninetieth day of life). Hume, Nunn, Smedley-MacLean and Smith ('38) found that the weight curve of rats receiving a low-fat diet began to exhibit a plateau after about 2 months. It was decided, therefore, to feed a low-fat diet and study the effect of various natural oils and unsaturated fatty acids upon the growth response of vitamin A-deficient rats to carotene.

#### EXPERIMENTAL

*Preparation of supplements.* Linseed oil, corn oil, wheat germ oil, soybean oil, butterfat, cottonseed oil, and coconut oil were used in feeding. The linseed oil,<sup>2</sup> corn oil,<sup>3</sup> wheat germ oil,<sup>4</sup> and soybean oil<sup>5</sup> were decolorized at room temperature by passage twice through columns packed with fuller's earth. The butterfat was decolorized in a similar manner, but was

<sup>2</sup> H. H. Rosenthal Co., Inc., New York, N. Y.

<sup>3</sup> Mazola brand, Corn Prod. Refining Co., Argo, Ill.

<sup>4</sup> Prepared by ether extraction of "Embo," General Mills, Inc., Minneapolis, Minn.

<sup>5</sup> Prepared by ether extraction of mature soybeans.

maintained at 50°C. during the process. The cottonseed oil<sup>6</sup> and coconut oil<sup>7</sup> were nearly colorless and were not further purified. The methyl linolate was prepared from corn oil and the methyl linolenate from linseed oil according to the methods of Rollett ('09 a, '09 b). The linoleic acid of corn oil was converted into the tetra-bromide and was purified by repeated recrystallizations from skellysolve (Skellysolve B, redistilled) until a beautifully crystalline material was obtained which melted sharply at 114.5° to 114.9°C. The linolenic acid of linseed oil was converted into the hexa-bromide and allowed to precipitate from glacial acetic acid. The hexa-bromide was recrystallized several times from benzene. The final product melted sharply at 179.3° to 179.5°C. Since it is well-known that the free unsaturated fatty acids deteriorate fairly rapidly, the major portion of these acids was stored as tetra- or hexa-bromides. Portions of the bromides were de-brominated every 2 or 3 weeks and esterified for feeding. Representative samples of methyl linolate and methyl linolenate prepared according to this procedure had iodine numbers (Wijs) of 161.0-162.3 and 241.2-242.0, respectively. Skellysolve solutions of methyl linolate and methyl linolenate containing 26.2 gm. of the esters/100 ml. were used in feeding. The usual daily dose of 0.2 ml. of these solutions supplied 0.05 gm. of the free acids. Solutions of carotene<sup>8</sup> in skellysolve containing 15-20 µg./ml. were used in feeding. It has been previously noted (Koehn and Sherman, '40) that solutions of carotene of this concentration are relatively stable; but to insure a constant intake, the exact concentration of carotene in the solutions was determined at bi-weekly intervals by spectrophotometric readings. This method of feeding carotene has an advantage over the usual method of feeding it in various oil solutions in that possible errors resulting from the rapid destruction of carotene in certain oils are completely eliminated. All supplements were stored in glass-stoppered bottles in the dark at 3°C.

<sup>6</sup> Wesson oil.

<sup>7</sup> Pu-re-co brand, Capital City Products Co., Columbus, Ohio.

<sup>8</sup> S.M.A. beta carotene.

*Feeding technique.* Prior to being placed upon the experimental supplement the rats (Wisconsin strain) were treated in a manner similar to the method used in this laboratory for vitamin A assay (Sherman, '40). When the young rats (usually eight to the litter) reached a weight of 40 to 50 gm. (23 to 26 days) they were transferred to individual cages and fed the vitamin A—test diet 31—A<sup>\*</sup> and distilled water ad libitum. No oil supplements were fed during the depletion period. The weekly weights of the rats were recorded during the first 4 weeks of the depletion period, after which the animals were weighed daily until growth had ceased and ophthalmia had developed (usually by the end of the fifth week of depletion). Weekly weights and observations of the gross appearance of the animals were recorded during the remainder of the experiments. In feeding the supplements, aliquots of the skellysolve solutions were pipetted each morning on about 2 gm. quantities of the basal diet contained in separate feed jars. When the skellysolve had evaporated, the contents of the jars were mixed by spatula after which the aliquot of oil (if fed) was added. The jars containing the supplements were immediately placed in the individual cages and the contents were usually consumed within 15 minutes. The animals receiving the various supplements were distributed with respect to sex and litters.

*Effect of oils upon the growth response to carotene.* The freedom of the oils from vitamin A activity is shown by the results obtained when they were fed without added carotene (table 1). The rats receiving 0.5 ml. of the oils daily continued to lose weight and within 5 weeks all died, exhibiting severe ophthalmia.

The growth of the rats receiving coconut oil and butterfat (0.1 ml. daily) with carotene at levels of 1 and 2 µg. daily was only slightly superior to that of control animals receiving carotene alone. Wheat germ oil, corn oil, linseed oil, and

<sup>\*</sup>Diet 31-A was composed of alcohol-extracted casein 18, salt mixture no. 186 (J. Biol. Chem., vol. 89, p. 199, 1930) 4, Northwestern yeast, irradiated 6, and sucrose 72%.

cottonseed oil definitely increased the growth. A still greater increase in growth was obtained with soybean oil at both levels of carotene intake.

*Effect of unsaturated fatty acids upon the growth response to carotene.* The most obvious difference between the oils which promoted growth and those which did not is the content

TABLE 1

*Effect of oils upon the growth response of vitamin A-deficient rats to beta carotene*

CAROTENE μg.	OIL <sup>1</sup>	NUMBER OF RATS	MOR- TALITY %	AVERAGE GAIN IN WEIGHT AT END OF				RANGE AT END OF 7TH WEEK gm.
				1st week gm.	3rd week gm.	5th week gm.	7th week gm.	
0.5	0.5 ml. coconut oil	2	100	-7	..	..	..	....
0.5	0.5 ml. wheat germ oil	2	100	-2	-17	..	..	....
0.5	0.5 ml. cottonseed oil	4	100	-2	-23	..	..	....
0.5	0.5 ml. linseed oil	4	100	0	-13	..	..	....
0.5	0.5 ml. soybean oil	3	100	-2	-21	..	..	....
1	.....	14	7	2	7	7	10	-12-23
1	0.1 ml. coconut oil	4	25	2	5	10	13	3-25
1	0.1 ml. butterfat	4	0	4	17	23	25	19-30
1	0.1 ml. linseed oil	9	0	10	29	38	45	25-80
1	0.1 ml. cottonseed oil	4	0	8	31	43	46	20-76
1	0.1 ml. soybean oil	4	0	10	36	55	69	42-92
2	.....	16	6	6	19	25	29	15-49
2	0.1 ml. coconut oil	6	16	4	15	24	30	13-41
2	0.1 ml. butterfat	4	25	3	12	25	37	32-42
2	0.1 ml. wheat germ oil	4	0	10	37	52	62	48-82
2	0.1 ml. corn oil	3	0	10	34	54	64	55-73
2	0.1 ml. linseed oil	4	0	8	32	57	70	62-80
2	0.1 ml. cottonseed oil	6	0	10	40	59	71	49-85
2	0.1 ml. soybean oil	12	0	13	42	68	91	73-128

<sup>1</sup> All oils except coconut and cottonseed oils were decolorized with fuller's earth.

of essential unsaturated fatty acids. The growth-promoting oils are all known to contain large amounts of linoleic acid; two of them, soybean and linseed oils, contain linolenic acid as well. Butterfat and coconut fat are low in these essential fatty acids. Moreover, the rats which received carotene alone and those which received carotene plus coconut oil or butter-

fat developed a dry scaliness of the skin typical of a deficiency of unsaturated fatty acids. There was definite improvement of the ophthalmic condition of the rats, but a slight swelling of the eyelids usually persisted throughout the entire duration of the experiment with both levels of carotene administration. The skin of the animals receiving the growth-promoting oils was normal in appearance and the ophthalmia was completely cured in nearly all of the rats.

It was decided, therefore, to feed methyl linolate and methyl linolenate to determine if these esters of unsaturated fatty acids would have growth-stimulating properties similar to those of the oils. The results, in the form of growth curves are summarized in figure 1. The growth response obtained when methyl linolate was fed with 2  $\mu\text{g}$ . of carotene was not only inferior to that obtained when the oils were fed with carotene but was invariably inferior to that obtained with carotene alone. The rats gained weight slowly for 2 or 3 weeks, then failed to gain, and finally lost weight throughout the remaining 2 or 3 weeks of the experiments. There was usually a slight improvement in the ophthalmia during the first 2 weeks, then a recurrence, and by the end of the experiments the rats usually exhibited severe ophthalmia. When fed methyl linolenate plus 2  $\mu\text{g}$ . of carotene the animals lost weight, exhibited severe ophthalmia and died before the end of the experiments. Vitamin A, in the form of a skellysolve solution of U.S.P. reference cod liver oil, also was fed with methyl linolate at a level to supply 3.3 U.S.P. XI units of vitamin A, equivalent to 2  $\mu\text{g}$ . of carotene. The methyl linolate had essentially the same antagonistic effect with vitamin A as with carotene, although the animals reached a slightly higher weight before starting to decline.

Because of the excellent growth obtained when carotene was supplemented with soybean oil, it was decided to feed soybean oil with methyl linolate and carotene to determine if it could counteract the antagonistic action of the methyl linolate. The addition of 0.1 ml. of soybean oil to the regular daily carotene

and methyl linolate supplements completely prevented the antagonism (fig. 1, curve E).

With the discovery that methyl linolate impaired the growth of rats on a low level of vitamin A intake, and that this harmful effect could be prevented by soybean oil, it was logical to

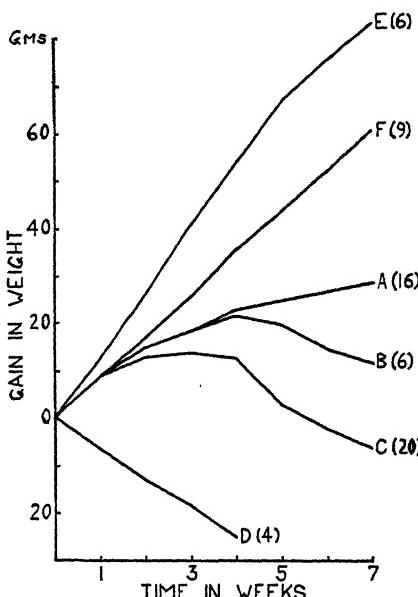


Fig. 1 Average growth curves for rats on low levels of carotene plus unsaturated fatty acids. The numbers in parentheses indicate the number of rats used in obtaining the averages. Curve A, 2 µg. carotene, range at end of experiment 15-49; curve B, 3.3 U.S.P. XI units of vitamin A + 0.05 gm. methyl linolate, range 4-32; curve C, 2 µg. carotene + 0.05 gm. methyl linolate, range -10-18; curve D, 2 µg. carotene + 0.05 gm. methyl linolenate, range -25-19; curve E, 2 µg. carotene + 0.05 gm. methyl linolate + 0.1 ml. soybean oil, range 53-104; curve F, 2 µg. carotene + 0.05 gm. methyl linolate fed about 6 hours apart, range 40-82.

determine whether impairment of growth was attributable to a chemical or physiological antagonism between the two substances, or to possible toxicity of the methyl linolate with soybean oil merely acting to decrease such toxicity. That the effect was not caused by toxicity of the methyl linolate was shown by experiments in which the methyl linolate and caro-

tene were fed separately a few hours apart. In these experiments, it was the general practice to feed the 2  $\mu$ g. of carotene early in the morning and the methyl linolate about 6 hours later. From the curve given in figure 1, it is apparent that the antagonism is largely prevented by this method of feeding.

Further evidence for an antagonism between carotene and methyl linolate was obtained by increasing the amount of carotene fed. The growth responses obtained when 5 and 10  $\mu$ g. of carotene were fed with 0.05 gm. of methyl linolate are shown in figure 2. With 5  $\mu$ g. of carotene, the methyl linolate

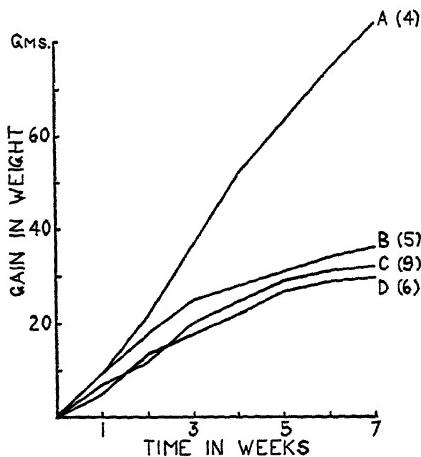


Fig. 2 Average growth curves for rats on high levels of carotene plus methyl linolate. The numbers in parentheses indicate the number of rats used in obtaining the averages. Curve A, 10  $\mu$ g. carotene + 0.05 gm. methyl linolate, range at end of experiment 58-104; curve B, 10  $\mu$ g. carotene, range 19-55; curve C, 5  $\mu$ g. carotene + 0.05 gm. methyl linolate, range 9-68; curve D, 5  $\mu$ g. carotene, range 15-48.

did not cause the rapid loss of weight which occurred with 2  $\mu$ g. of carotene; but there was no growth stimulation. There was sufficient antagonism to cause a recurrence of ophthalmia in a number of the rats by the seventh week. When 10  $\mu$ g. of carotene was fed, the addition of 0.05 gm. of methyl linolate gave a definite growth stimulation and there was no evidence of ophthalmia in the animals.

*Effect of methyl linolate upon the stability of carotene.* The above results suggested the possibility that the carotene was being destroyed by an oxidation-reduction reaction with the methyl linolate before being consumed by the rats. Evidence of such a reaction has been obtained by Monaghan and Schmitt ('32). To test this possibility, varying amounts of carotene and methyl linolate were mixed with small amounts of the basal diet and exposed to the air in thin layers for periods of 1.5 to 24 hours at 25, 28 and 37°C. The carotene and methyl linolate were then thoroughly extracted with skelly-solve. The extract was saponified and analyzed for carotene

TABLE 2  
*Effect of methyl linolate upon carotene stability*

CAROTENE ADDED	ME. LINOLATE ADDED	TIME	TEMPERATURE	CAROTENE RECOVERED
μg.	gm.	hours	°C.	μg.
25.8	....	3	25	24.2
25.8	0.20	3	25	24.8
45.2	....	1.5	28	44.8
45.2	0.10	1.5	28	45.2
45.2	....	24	25	30.2
45.2	0.10	24	25	30.6
21.8 <sup>1</sup>	....	3	37	16.9
21.8 <sup>1</sup>	0.20	3	37	21.7

<sup>1</sup>In the last experiment the carotene and methyl linolate were incubated with the stomach contents of normal rats.

according to a previously described method (Sherman and Salmon, '39). To determine if gastric acidity were a factor, a test was carried out in which the carotene and methyl linolate were incubated with the gastric contents of normal rats. The results summarized in table 2 show that there was no evidence of direct destruction of the carotene by the methyl linolate; instead, in all instances the methyl linolate apparently exerted a slight protective influence.

Carotene recovered after incubation with methyl linolate was further examined spectrophotometrically and was found to have the same absorption curve as the original carotene. Carotene incubated with methyl linolate was also tested bio-

logically to determine if there were any changes which could not be detected chemically. In this experiment, 636 µg. of carotene and 5.0 gm. of methyl linolate were incubated at 25°C. for 3 hours. The recovered carotene (630 µg.) was then fed to vitamin A-depleted rats at a level of 2 µg. with 0.1 ml. of soybean oil daily. The results given in table 3 show that there was no loss in the biological activity of the carotene by treatment with methyl linolate.

TABLE 3  
*Biological value of carotene as affected by exposure to methyl linolate*

DAILY SUPPLEMENT	NUMBER OF RATS	AVERAGE GAIN IN WEIGHT AT END OF				RANGE AT END OF 7TH WEEK
		1st week	3rd week	5th week	7th week	
		gm.	gm.	gm.	gm.	gm.
2 µg. exposed carotene <sup>1</sup> + 0.1 ml. soybean oil	3	15	38	70	86	79-92
2 µg. untreated carotene + 0.1 ml. soybean oil	3	16	42	72	87	71-99

<sup>1</sup> The carotene was allowed to remain in an open jar with methyl linolate for 180 minutes at 25°C.

*Effect of oils and methyl linolate upon carotene absorption.* Several investigators (Basu, '37; Wilson et al., '36) have attributed variations in the potency of carotene or vitamin A dissolved in various oils to differences in the intestinal absorption of carotene or vitamin A. That mineral oil causes a lowered absorption of carotene is well known. Carotene analyses were therefore made on the feces of rats receiving carotene alone and carotene plus oils or methyl linolate to determine if the difference in the responses obtained could be explained by the effect of the oils upon the absorption of carotene. For these analyses, the fecal material of each rat was collected daily for 7 days and stored at 3°C. under methanol. The feces were then finely ground and analyzed for carotene (Sherman and Salmon, '39). The results from these analyses are summarized in table 4. It is apparent that a relatively efficient absorption of carotene, fed at a level of 5 µg. daily, was obtained regardless of the nature of the oil supplements, al-

though there was slightly more efficient absorption with all of the oils than with the carotene alone. The results, however, do not explain the differences in the growth responses obtained with the various oils and methyl linolate.

TABLE 4  
*Effect of fats upon carotene absorption by rats*

DAILY SUPPLEMENT	NUMBER OF RATS	CAROTENE FED	FECAL CAROTENE <sup>1</sup>	CAROTENE ABSORBED
5 µg. carotene	12	µg. 35	µg. 5.9	% 83.3
5 µg. carotene + 0.1 ml. coconut fat	8	35	3.9	88.6
5 µg. carotene + 0.1 ml. linseed oil	8	35	4.0	88.5
5 µg. carotene + 0.1 ml. soybean oil	4	35	4.4	87.5
5 µg. carotene + 0.05 gm. methyl linolate	5	35	4.6	87.0

<sup>1</sup> Values for fecal carotene were corrected for the pigment excreted by control rats not receiving carotene (an average of 1.5 µg., calculated as carotene, in 7 days).

#### DISCUSSION

The results of these experiments demonstrate that with a low-fat diet the response of vitamin A-deficient rats to carotene is influenced by the nature of the oil fed with the carotene. It appears probable that the disagreement between these findings and those of Lease, Lease, Steenbock and Baumann ('39) can be attributed to differences in the fat content of the basal diets used. These workers obtained approximately equal growth responses when carotene was fed with cottonseed oil, soybean oil, lard, decolorized butterfat, coconut oil, or peanut oil at a 3% level to rats which had been depleted of vitamin A on a basal diet containing 10% fat. The growth-stimulating power of the oils seems to be partially dependent upon the content of essential unsaturated fatty acids. However, the feeding of methyl linolate and methyl linolenate with low levels of carotene or vitamin A has revealed the existence of

a very interesting antagonistic relationship. This antagonism is characterized by a retarded growth rate and an incomplete cure of ophthalmia. It appears that the unsaturated fatty acids interfere with some phase of the metabolism of carotene or vitamin A since the harmful effect of the methyl linolate is largely overcome by feeding the two materials a few hours apart, during which time the animals are apparently able to convert either the free linoleic acid or carotene into some less reactive form. That this antagonism does not appear when natural oils high in linoleic acid content are fed is possibly attributable to the presence of some other factor or factors in the oils which counteract this antagonism.

On the basis of these findings, it would appear advisable for investigators who are employing unsaturated fatty acids in feeding to give the vitamin A supplement separately to insure its effective utilization.

#### SUMMARY

Various natural oils were fed to vitamin A-deficient rats receiving controlled levels of carotene. Of the oils tested, soybean oil gave the best growth. Cottonseed oil, linseed oil, corn oil, and wheat germ oil also had a beneficial effect upon the growth. Butterfat and coconut oil had no appreciable effect.

Tests with methyl linolate and methyl linolenate revealed an antagonistic action when fed with low levels of carotene. When sufficient carotene was fed with the methyl linolate, this antagonism was overcome and the methyl linolate gave a definite growth stimulation. This antagonism was counteracted at the low level of carotene intake by the addition of soybean oil and also by feeding the carotene and methyl linolate a few hours apart.

Carotene analyses of the feces of the rats showed that the differences in the growth response to various oils and methyl linolate could not be explained on the basis of differences in the effect of the oils on the absorption of carotene.

## LITERATURE CITED

- BASU, N. K. 1937 Vitamin A and fat metabolism. *Z. Vitaminforsch.*, vol. 6, p. 106.
- BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by the rigid exclusion of fat from the diet. *J. Biol. Chem.*, vol. 82, p. 345.
- BURR, G. O., M. M. BURR AND E. S. MILLER 1932 On the fatty acids essential in nutrition. *J. Biol. Chem.*, vol. 97, p. 1.
- DE, N. K. 1937 The assimilation of vitamin A and carotene by rats from some common foods with a note on the conversion factor, I.U./E., proposed by the International Vitamin Conference. *Indian J. Med. Research*, vol. 24, p. 751.
- HUME, E. M., L. C. A. NUNN, I. SMEDLEY-MACLEAN AND H. H. SMITH 1938 Studies of the essential unsaturated fatty acids in their relation to the fat-deficiency disease of rats. *Biochem. J.*, vol. 32, p. 2162.
- KOEHN, C. J., AND W. C. SHERMAN 1940 The determination of vitamin A and carotene with the photoelectric colorimeter. *J. Biol. Chem.*, vol. 132, p. 527.
- KRAYBILL, H. R., AND C. L. SHREWSBURY 1936 The relative vitamin A potency of carotene fed in butter fat and cottonseed oil. *J. Nutrition*, vol. 11, p. 103.
- LATHBURY, K. C., AND G. N. GREENWOOD 1934 The influence of the solvent on the biological effect of carotene and vitamin A. *Biochem. J.*, vol. 28, p. 1665.
- LEASE, E. J., J. G. LEASE, H. STEENBOCK AND C. A. BAUMANN 1939 The biological value of carotene in various fats. *J. Nutrition*, vol. 17, p. 91.
- MONAGHAN, B. R., AND F. O. SCHMITT 1932 The effects of carotene and of vitamin A on the oxidation of linoleic acid. *J. Biol. Chem.*, vol. 96, p. 387.
- ROLLETT, A. 1909 a Linoleic acid. *Z. Physiol. Chem.*, vol. 62, p. 410.  
\_\_\_\_\_. 1909 b Linolenic and linseed oil. *Z. Physiol. Chem.*, vol. 2, p. 422.
- SHERMAN, W. C., AND W. D. SALMON 1939 Carotene content of different varieties of green and mature soybeans and cowpeas. *Food Research*, vol. 4, p. 371.
- SHERMAN, W. C. 1940 Chromatographic identification and biological evaluation of carotene from mature soybeans. *Food Research*, vol. 5, p. 13.
- WILSON, H. E. C., B. AHMAD AND B. N. MAJUMDAR 1936 Further observations on the metabolism of carotene. *Indian J. Med. Research*, vol. 24, p. 399.



# THE VALUE OF UREA IN THE SYNTHESIS OF PROTEIN IN THE PAUNCH OF THE RUMINANT

## I. IN MAINTENANCE<sup>1</sup>

LORIN E. HARRIS AND H. H. MITCHELL

*Division of Animal Nutrition, University of Illinois, Urbana*

TWO FIGURES

(Received for publication February 17, 1941)

In 1911, Armsby reviewed the literature on the subject of the synthesis of protein from non-protein compounds in the alimentary canal of animals, and concluded (1) that there was no evidence that the monogastric animal could utilize the non-protein compounds in this way, but (2) that with polygastric animals the synthesis by the micro-organisms of the paunch is demonstrable but quite limited in extent. The literature was again reviewed by Mitchell and Hamilton in 1929 to essentially the same effect. A third critical review of the literature on the subject was published by Krebs in 1937. While admitting that the evidence indicated some protein sparing effect of such compounds as urea and glycocoll, Krebs was reluctant to believe that any considerable protein synthesis resulted, or that, if it did result, the bacterial protein produced was of any appreciable value to the host.

However, experiments published since the review of Krebs have supplied convincing evidence that urea and ammonium salts may, to a considerable extent, serve the functions of protein in the ruminant. The nitrogen balance experiments of Fingerling and his associates ('37) are clear-cut, while the

<sup>1</sup> The data reported in this paper were taken from a thesis submitted by Lorin E. Harris to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Husbandry, August, 1940.

growth experiments of Bartlett and Cotton ('38), and particularly of Hart, Bohstedt, Deobald and Wegner ('39) furnish good evidence that low-protein rations, inadequate for the growth of calves, are effectively supplemented by urea. And finally attention may be called to the excellent work of Studt ('39) who, using an experimental method analogous to that employed by McElroy and Goss ('39, '40 a, '40 b) in demonstrating vitamin synthesis in the paunch, showed that in sheep subsisting on a low-protein amide-containing ration there is an accumulation of true protein in the paunch 18 hours after the last feeding.

The experiments described below were undertaken with the conviction that a considerable conversion of urea into protein in the paunch has been demonstrated. The main purpose of the experiments was, first, to determine whether the rate of conversion was sufficient to cover the maintenance requirements of sheep, and, second, to measure the efficiency of conversion by determining the biological value of urea in comparison with that of casein.

#### PLAN OF THE EXPERIMENTS

The experiment consisted of a series of nitrogen balance studies on eight grade sheep, mainly of Rambouillet breeding, ranging from 15 to 18 months in age and from 27 to 40 kg. in body weight. The endogenous urinary nitrogen and the metabolic fecal nitrogen were determined on all sheep by measuring the output of nitrogen in urine and feces after subsistence upon a low-nitrogen ration until the output of urinary nitrogen and the percentage of creatinine nitrogen on the total nitrogen became constant from day to day. Then the nitrogen metabolism was studied in periods of 6 to 34 days in length during which the basal ration was supplemented with graded amounts of urea or casein.

In order to test the finding of Scheunert, Klein and Steuber ('22) that ingested urea is in considerable part excreted through the skin of sheep, all of the sheep in this experiment were washed thoroughly before and after each collection period

with a warm solution of soap and sodium carbonate and rinsed with about 17 liters of distilled water. To facilitate this treatment, the sheep were sheared prior to the initiation of the metabolism experiments. During these experiments the sheep were confined in metabolism crates of a design similar to that described by Forbes ('15).

The low-nitrogen basal ration was modified frequently at the start of the experiments in order to secure a mixture of satisfactory palatability. That used in most of the experiments contained in per cent: wheat straw 25.0, wood pulp 10.5, corn starch 28.42, brown sugar 29.0, corn oil 4.5, complete salt mixture 2.0, and citric acid 0.58. The mixture was dextrinized by steam cooking with water. It was then dried and broken into small lumps. In this form it contained on an average 95.71% of dry matter, 0.136% nitrogen and 4.016 calories of gross energy per gram. In compounding the other rations, urea or casein would be either incorporated in the above ration or, in the case of urea, added to the day's ration. Each sheep received daily, in addition to its solid food, 2 cc. of a fortified cod liver oil.

The food intakes of the sheep were maintained constant insofar as possible throughout a series of comparable metabolism periods, though frequently in a final low-nitrogen period the appetite of the sheep would decline and food consumption would decrease.

All ration mixes, urine collections and feces collections were analyzed for total nitrogen by the Kjeldahl method and the urines were analyzed for creatine and creatinine essentially by the method of Folin ('14) using purified picric acid as described by Benedict ('29) and substituting the photoelectric colorimeter for the DuBosq colorimeter. Cellulose and lignin were determined in food and feces by the methods of Crampton and Maynard ('38).

#### DISCUSSION OF EXPERIMENTAL RESULTS

*The endogenous urinary nitrogen.* In twenty metabolism periods on the eight experimental sheep on the low-nitrogen

basal ration, the endogenous nitrogen in the urine averaged 0.0393 gm. daily per kilogram of body weight, or 1.425 gm. per  $m^2$  of body surface (Ritzman and Colovos, '30). Eliminating four high results after the application of Chauvenet's criterion, reduces the average to 0.0333 gm. per kilogram of body weight, or 1.231 gm. per  $m^2$  of body surface daily, the coefficients of variation being 9.43 and 10.40, respectively. The value of 1.231 gm. of endogenous nitrogen per  $m^2$  of body surface is considerably smaller than similar values found by Smuts ('35) for the mouse, rat, guinea pig, rabbit or pig. The value of 0.0333 gm. per kilogram body weight agrees very well with the average of 0.035 gm. for sheep reported by Smuts and Maraais ('38), that of 0.037 gm. reported by Miller and Morrison ('39), and also that of 0.0331 gm. obtained by Sotola ('30).

The creatinine nitrogen averaged 24.7% of the total urinary nitrogen in the low-nitrogen periods and the average creatinine coefficient was 22.5. Creatine was present in all of these urines to the extent of 0.032 to 0.244 gm. daily.

*The metabolic nitrogen of the feces.* In the twenty low-nitrogen periods, the fecal nitrogen excretion averaged 0.555 gm. per 100 gm. of dry matter consumed, with a coefficient of variation of 11.91. This value is identical with that obtained with sheep by Miller and Morrison ('39) and approximates the earlier value of Morgen, Beger and Westhauser (cited by Mitchell, '26), i.e., 0.51 gm. of metabolic nitrogen per 100 gm. of dry matter consumed. It is considerably less than Sotola's ('30) average of 0.66 gm.

*The nutritive effects of urea and casein.* The appetites of the animals always improved on the transition from the low-nitrogen basal ration to the urea-supplemented or the casein-supplemented rations, and weight maintenance was more readily secured.

The finding of Scheunert, Klein and Steuber ('22) that urea is excreted through the skin of sheep, based upon three washings on one sheep, was not confirmed. The averages collected in table 1 show that the amount of nitrogen contained in the

TABLE 1

*Average daily amounts of nitrogen in the wool washings for the different experimental rations*

RATION	NUMBER OF WASHINGS	AVERAGE LENGTH OF PERIODS	NITROGEN IN WASHINGS	
			Average per day	AVERAGE PER DAY PER KG.M. BODY WEIGHT
		days	gm.	gm.
Normal	7	44.1	0.133	0.0037
Casein	7	16.3	0.146	0.0039
Urea	19	21.1	0.112	0.0032
N-low	19	12.8	0.114	0.0036

wool washings is unaffected by the type of ration consumed and is low in all cases. It probably represents a rather constant excretion through the skin, together with urine contamination from the floor of the metabolism cage. In the computations of nitrogen balance reported below, the washings nitrogen has been taken into account as a component of the nitrogen excretion.

The fecal nitrogen increased in the transition from a low-nitrogen ration to one containing either casein or urea. The coefficients of apparent and true digestibility for the casein and urea nitrogen are summarized in table 2. The average true digestibility of casein nitrogen, the metabolic nitrogen

TABLE 2  
*Average digestion coefficients for casein and urea nitrogen*

SHEEP NO.	DIGESTIBILITY OF CASEIN NITROGEN			DIGESTIBILITY OF UREA NITROGEN		
	Number of tests	Apparent digesti- bility	True digesti- bility	Number of tests	Apparent digesti- bility	True digesti- bility
3	1	23.3	82.5	1	24.3	85.6
4	..	...	...	1	23.0	86.0
5	2	38.8	90.6	5	45.9	91.0
6	2	40.4	94.8	4	34.4	81.7
7	..	...	...	4	44.0	89.4
8	..	...	...	4	54.2	93.5
9	..	...	...	1	24.5	99.2
10	2	49.3	79.6	5	51.3	83.0
Average		38.0	86.9		37.7	88.8

of the feces being duly considered, was 86.9%, while that for urea nitrogen was 88.8%. The fact that the nitrogen of a readily soluble compound like urea is not completely digested by the sheep, is consistent with the hypothesis that it has been converted into bacterial protein in the paunch.

The digestibility of dry matter in the basal ration was distinctly improved by the urea supplements (table 3), while the increases observed with the casein supplements can be accounted for only in part by a possible greater digestibility of casein than of the dry matter of the basal ration. Of the dry matter constituents of the basal ration, the cellulose was

TABLE 3  
*Average digestion coefficients for dry matter and cellulose*

SHEEP NO.	DIGESTIBILITY OF DRY MATTER						DIGESTIBILITY OF CELLULOSE			
	Casein ration		Urea ration		N-low ration		Urea ration		N-low ration	
	No. of tests	Digesti- bility	No. of tests	Digesti- bility	No. of tests	Digesti- bility	No. of tests	Digesti- bility	No. of tests	Digesti- bility
5	2	64.1	5	66.7	3	62.5	2	42.7	2	31.0
6	2	67.8	4	64.8	5	59.0	1	43.9	4	10.0
7	..	...	4	66.7	2	59.8	2	30.0	2	13.2
8	..	...	4	65.2	2	63.3	4	36.5	2	23.8
9	..	...	1	64.8	2	62.8	1	34.9	2	25.0
10	2	68.7	5	66.3	3	59.1	2	44.4	2	3.8
Average		66.9		65.8		61.1		38.7		17.8

digested to a much greater extent (38.7%) in the rations containing urea than in the unsupplemented basal ration (17.8%). The digestibility of lignin was not affected by urea, and data on this point are omitted from table 3. The increased digestion (disappearance) of dry matter and of cellulose contained in the basal ration is consistent with the hypothesis that the addition of urea to such a ration stimulates the proliferation of the paunch flora and enhances carbohydrate fermentation there.

The nitrogen balance, consistently negative on the low-nitrogen regime, was always improved when either casein or urea was incorporated in the ration. Furthermore, it was

always possible with either casein or urea to bring the sheep into nitrogen equilibrium or even to promote a positive balance of nitrogen by using large enough supplements of these substances. These facts were demonstrated with all of the experimental sheep, but the data will not be presented in detail. The relation between the nitrogen intakes of the sheep per kilogram of body weight and the balance of nitrogen expressed on the same basis for all of the sheep for which sufficient data were obtained is depicted graphically in the charts. The relationship is evidently rectilinear, or approximately so, within the range of nitrogen intake that prevailed.

A typical series of nitrogen balances on sheep 7 is given in table 4.

TABLE 4

*A series of nitrogen metabolism studies on sheep 7. Results expressed on the daily basis*

RATION	LENGTH OF PERIOD	DRY MATTER EATEN	NITROGEN INTAKE		NITROGEN OUTPUT			NITROGEN BALANCE
			In basal ration	In supple- ment	In feces	In urine	In wash- ings	
Low-nitrogen	days	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Low-nitrogen	11	371	0.50	0	1.86	0.89	0.07	-2.32
Urea	10	381	0.55	2.32	2.11	1.06	0.06	-0.36
Low-nitrogen	8	302	0.45	0	1.49	0.86	0.07	-1.97
Urea	34	424	0.62	4.63	2.68	1.83	0.06	+0.73

For all sheep, the basal ration provided only from 10 to 30% of the endogenous loss of nitrogen, averaging 22.4%, except in two periods with sheep 5, 6 and 10, when a sample of wheat straw much higher in nitrogen than usual was inadvertently included in the basal ration.

The significance of the nitrogen balances may be questioned on the grounds, either (1) that there is a loss of ammonia nitrogen in the expired air, since urea is rapidly converted to ammonia in the paunch (Lenkeit and Becker, '38), or (2) that there is an accumulation of urea in the blood of ruminants fed this compound. The first possibility was explored with negative results by Scheunert, Klein and Steuber ('22). Wegner, Booth, Bohstedt and Hart ('40) could detect no loss of nitro-

gen from an incubating mixture of rumen bacteria and urea. The absorption of ammonia from the paunch and its excretion through the lungs seems impossible, since the lungs are impermeable to ammonia (Magnus, '02). The second possibility was studied by Bartlett and Cotton ('38) and by Hart and associates ('39) with negative results.

Confirming the significance of the nitrogen balance data relative to the efficacy of urea in covering the nitrogen requirements of maintenance, are the protracted experiments on sheep 7 and 8. The body weights of these sheep were maintained for 109 and 122 days respectively on the low-nitrogen basal ration with additions of urea sufficient to establish a small positive nitrogen balance. At the end of these periods, the health and appetite of the sheep were good and the experiment probably could have been continued indefinitely.

*The amounts of urea and casein nitrogen required for equilibrium.* The relation between the daily intakes of nitrogen and the daily balances of nitrogen for the various experimental periods and sheep, both quantities expressed per kilogram of body weight, are shown graphically in charts 1 and 2. The data for urea were sufficiently numerous for sheep 5, 6, 7, 8 and 10 to warrant individual presentation. A composite diagram for these sheep, including also the less complete data for sheep 3, 4 and 9, is also presented. The data for casein are composited in one diagram.

The relationship appears to be at least roughly rectilinear and hence in each diagram a rectilinear equation was fitted to the data by the method of least squares. The intersection of this line with the abscissa representing equilibrium locates the intake of nitrogen required for equilibrium. Except for the small amounts of nitrogen in the basal ration, equivalent to about 20 mg. daily per kilogram of body weight, these intakes of nitrogen required for equilibrium represent either urea nitrogen or casein nitrogen. For the five sheep with sufficient data to warrant this statistical treatment, the amounts of urea nitrogen necessary for equilibrium were 215 mg. per kilogram of body weight daily for sheep 5, 248 mg. for sheep 6, 122 mg.

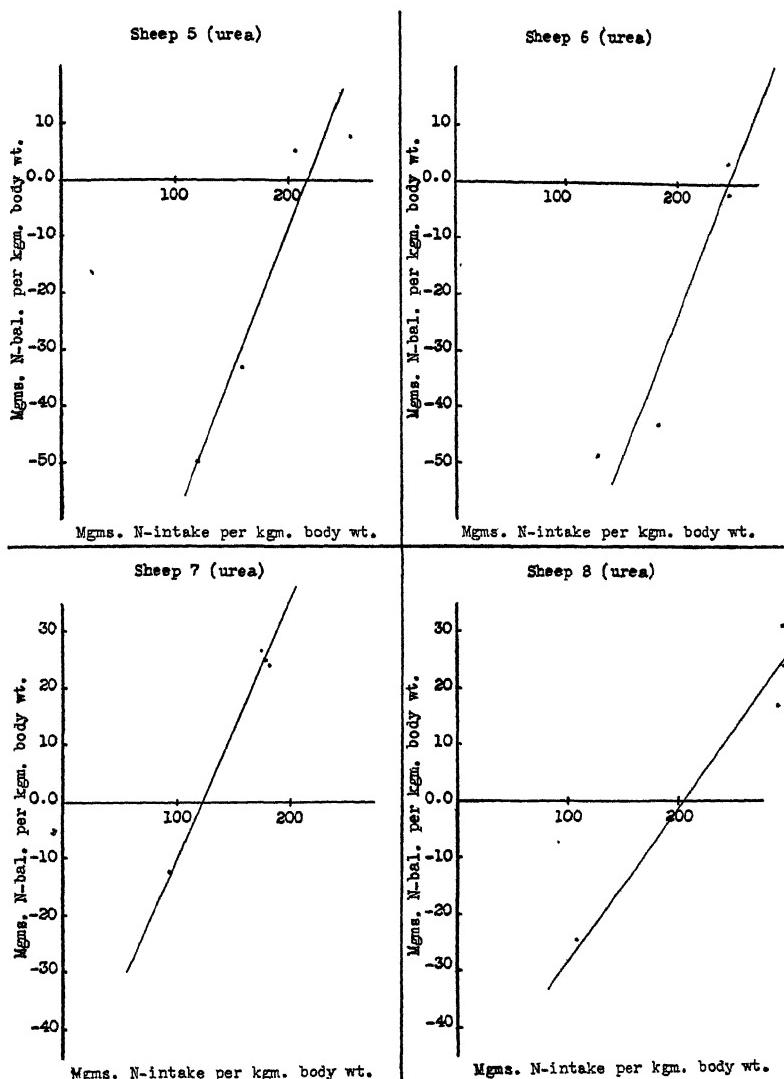


Fig. 1 The relation between the amounts of urea nitrogen and casein nitrogen consumed daily and the nitrogen balance.

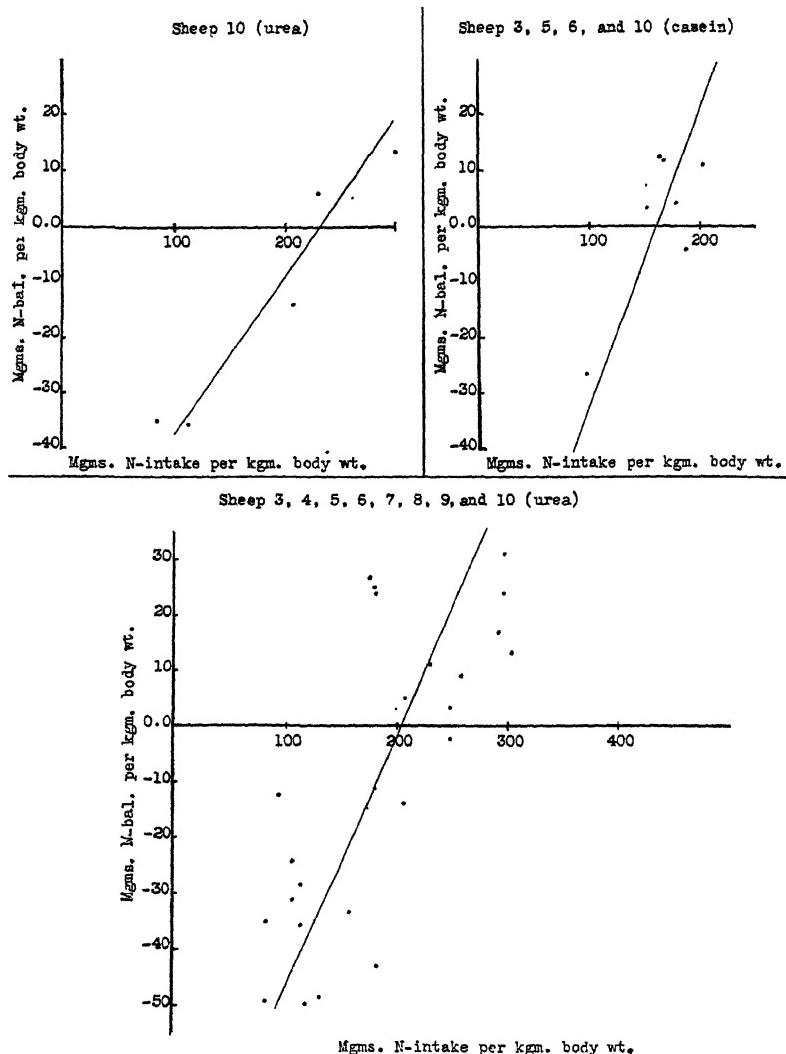


Fig. 2 The relation between the amounts of urea nitrogen and casein nitrogen consumed daily and the nitrogen balance.

for sheep 7, 204 mg. for sheep 8 and 229 mg. for sheep 10. The greater efficiency of sheep 7 in this respect is clearly evident from chart 1. The average of the above values is 204 mg., and the intercept in the composite diagram, containing also less complete data for three other sheep, is 202 mg.

From the composite diagram for the casein experimental periods an intercept of 161 mg. per kilogram of body weight is obtained. A comparison of the two average values, 202 mg. for urea nitrogen and 161 mg. for casein nitrogen, indicates that the former is only 80% as valuable as the latter in the replacement of the losses of endogenous nitrogen in the sheep.

Klein, Schmid, Studt and Müller ('39), from metabolism experiments on five sheep weighing about 50 kg. and subsisting on a ration of molasses, straw, chaff, starch, sucrose, lactose and salt, calculated a physiological protein minimum of 410 mg. of digestible protein per kilogram of body weight necessary for nitrogen equilibrium. This value was obtained by multiplying the urinary nitrogen at equilibrium by 6.25. It relates mainly to the amide nitrogen of molasses which provided most of the nitrogen consumed. In terms of nitrogen, the above value is equivalent to 66 mg.

The values obtained in this study refer to the nitrogen intake, and include not only the indigestible nitrogen of the feces but also the metabolic nitrogen. To obtain values comparable to that of Klein and his associates, they must be multiplied by 0.38, since the apparent digestibility of the dietary nitrogen was 38% (table 2). The product of  $202 \times 0.38$  is 78 mg., and that of  $161 \times 0.38$  is 61 mg. The value of Klein et al., i.e., 66 mg., is almost midway between the two values thus computed.

*The biological values of urea nitrogen and casein nitrogen for maintenance.* Biological values for urea nitrogen and casein nitrogen were calculated in the usual way (Mitchell, '24). The results of these calculations, arranged in the order of increasing levels of dietary nitrogen, are summarized in table 5.

TABLE 5  
*Estimations of biological values of urea and casein nitrogen*

SHEEP NO.	DAILY NITROGEN INTAKE			DAILY NITROGEN BALANCE	BIOLOGICAL VALUE
	Amount	Multiple of N requirement <sup>1</sup>	Protein equivalent in ration <sup>2</sup>		
Urea					
	gm.		%	gm.	%
4	2.22	0.56	4.13	-1.48	99
10	2.34	0.66	3.40	-1.22	89
3	2.84	0.89	4.23	-0.99	92
Average	2.47	0.70	3.92	-1.23	93
5	3.14	1.00	4.55	-1.57	44
6	3.07	1.00	4.47	-1.42	67
7	2.32	1.00	3.80	-0.37	92
8	2.75	1.00	4.46	-0.77	79
9	2.75	1.00	3.96	-0.86	75
10	3.51	1.00	5.09	-1.29	60
Average	2.92	1.00	4.39	-1.05	70
5	4.19	1.33	6.09	-1.02	43
7	4.63	2.00	6.83	+0.73	76
5	7.44	2.49	8.06	+0.20	60
6	8.37	2.82	9.91	+0.03	54
8	8.25	3.00	11.91	+0.71	49
5	9.30	3.11	10.12	+0.31	49
10	8.33	3.37	9.57	+0.47	42
10	9.59	4.44	12.65	+0.54	36
Casein					
3	2.43	0.76	4.26	-0.76	100
5	5.31	1.78	5.79	+0.13	87
6	5.31	1.79	6.32	+0.42	88

<sup>1</sup> Multiple of negative nitrogen balance on low-nitrogen ration.

<sup>2</sup> ( $N \times 6.25$ ) expressed as a percentage of the dry matter, disregarding the small amount of nitrogen in the basal ration.

The results show the anticipated drop in the biological value of urea nitrogen with increasing levels of intake. They also reveal for urea an unusually wide range of values for the same level. The exceptionally high biological values obtained consistently for sheep 7 are particularly notable. Since the utilization of urea is a function of the paunch flora and fauna, the variation in utilization would be associated with variations from one animal to another and from one time to another.

in the types of micro-organisms present in the paunch and in the conditions existing there bearing on the development and propagation of bacteria and protozoa.

At the lowest levels of intake the urea nitrogen seems to be practically completely utilized in metabolism. At the same concentrations of dietary nitrogen, the nitrogen of casein seems to be definitely better utilized than that of urea.

It is possible to compute the biological values of the nitrogen of urea and casein at the precise point of nitrogen equilibrium from the data given in the last section relative to the amounts of urea and casein nitrogen, respectively, required for equilibrium, i.e., 202 and 161 mg. per day per kilogram of body weight. This computation is made according to the following formula:

$$B = \frac{E}{ID - \frac{M}{B}}$$

in which B is the biological value expressed as a decimal, E is the endogenous nitrogen, I the nitrogen intake required for equilibrium, M the metabolic nitrogen, and D the true digestibility of the dietary N, expressed as a decimal. Per kilogram of body weight per day, E = 33.3 mg. and M = 77.4 mg., I = 202 mg. for urea nitrogen and 161 mg. for casein nitrogen and D = 0.888 for urea nitrogen and 0.869 for casein.

Thus computed, the average biological values of urea and casein nitrogen at nitrogen equilibrium are 62 and 79, respectively.

#### CONCLUSIONS

1. The endogenous nitrogen excretion of sheep weighing about 34 kg. and ranging in age from 15 to 18 months averages 0.0333 gm. per kilogram of body weight and 1.23 gm. per m<sup>2</sup> of body surface. The metabolic nitrogen in the feces averages 0.55 gm. per 100 gm. of dry matter consumed.

2. Urea administered to sheep in amounts commensurate with their requirements for maintenance is not excreted through the skin.

3. Urea added to a low-nitrogen ration improves the digestibility of cellulose markedly and is itself digested to the extent of 88.8%.

4. Sheep may be maintained in body and nitrogen equilibrium for well over 100 days on rations containing urea and minimal amounts of protein providing only one-tenth the amount of nitrogen needed for equilibrium.

5. Nitrogen equilibrium may be maintained on 202 mg. of urea nitrogen and 161 mg. of casein nitrogen per kilogram of body weight daily.

6. At nitrogen equilibrium, the biological value of urea nitrogen is 62, and of casein nitrogen 79.

The authors wish to acknowledge the invaluable advice and assistance of Dr. W. G. Kammlade and Mr. W. J. Hampton of the Sheep Husbandry Division in the feeding and care of the sheep used in these experiments.

#### LITERATURE CITED

- ARMSBY, H. P. 1911 The nutritive value of the nonprotein of feeding stuffs. U. S. Dept. Agr., Bur. An. Ind., Bul. 139, p. 49.
- BARTLETT, S., AND A. G. COTTON 1938 Urea as a protein substitute in the diet of young cattle. *J. Dairy Res.*, vol. 9, pp. 263-272.
- BENEDICT, S. R. 1929 A note on the purification of picric acid for creatinine determination. *J. Biol. Chem.*, vol. 82, pp. 1-3.
- CRAMPTON, E. W., AND L. A. MAYNARD 1938 The relation of cellulose and lignin content to the nutritive value of animal feeds. *J. Nutrition*, vol. 15, pp. 383-396.
- FINGERLING, G., B. HIENTZSCH, H. KUNZE AND K. REIFGERST 1937 Ersatz des Nahrungsgeiweisses durch Harnstoff beim wachsenden Rinde. *Landw. Vers.-Stat.*, vol. 128, pp. 221-235.
- FOLIN, O. 1914 On the determination of creatinine and creatine in the urine. *J. Biol. Chem.*, vol. 17, pp. 469-473.
- FORBES, E. B. 1915 A metabolism crate for swine. *Ohio Agr. Exp. Sta. Circ.* 152, pp. 75-85.
- HART, E. B., G. BOHSTEDT, H. J. DEOBALD AND M. I. WEGNER 1939 The utilization of simple nitrogenous compounds such as urea and ammonium bicarbonate by growing calves. *J. Dairy Sci.*, vol. 22, pp. 785-798.

- KLEIN, W., H. SCHMID, E. STUDT AND R. MÜLLER 1939 Der Strukturwert des aus nichteiweissartigen Verbindungen im Pansen erzeugten Bakterieneiweisses auf Grund des festgestellten physiologischen Eiweissminimums. *Z. Tierzücht. u. Züchtungsbiol.*, vol. 43, pp. 76-110.
- KREBS, K. 1937 Der Wert der Amide bei der Fütterung des Rindes. Historische Betrachtung der Entwicklung der Amidfrage, Kritische Wertung des Standes unserer heutigen Kenntnisse. *Biedermann's Zentralbl. Agriculturnchem. Abt. B. Tierernähr.*, vol. 9, pp. 394-507.
- LENKEIT, W., AND M. BECKER 1938 Das Schicksal des Harnstoffs der Amidflocken im Pansen. *Zeit. Tierernähr. in Futtermittelkunde*, vol. 1, pp. 97-101.
- MAGNUS, E. 1902 Ueber die Undurchgängigkeit der Lunge für Ammoniak. *Arch. exp. Pathol. u. Pharm.*, vol. 48, pp. 100-106.
- MC ELROY, L. W., AND H. GOSS 1939 Report on four members of the vitamin B complex synthesized in the rumen of the sheep. *J. Biol. Chem.*, vol. 130, pp. 437-438.
- 1940 a A quantitative study of vitamins in the rumen contents of sheep and cows fed vitamin-low diets. I. Riboflavin and vitamin K. *J. Nutrition*, vol. 20, pp. 527-540.
- 1940 b A quantitative study of vitamins in the rumen contents of sheep and cows fed vitamin-low diets. II. Vitamin B<sub>6</sub> (pyridoxine). *J. Nutrition*, vol. 20, pp. 541-550.
- MILLER, J. I., AND F. B. MORRISON 1939 Relative efficiency for growing lambs of the protein in rations containing alfalfa hay, timothy hay, and combinations of the two hays. *J. Agr. Res.*, vol. 58, pp. 149-155.
- MITCHELL, H. H. 1924 A method of determining the biological value of protein. *J. Biol. Chem.*, vol. 58, pp. 873-903.
- 1926 The determination of the protein requirements of animals and of the protein values of farm feeds and rations. *Bull. Natl. Res. Council* no. 55, 44 pp.
- MITCHELL, H. H., AND T. S. HAMILTON 1929 The biochemistry of the amino acids. *Am. Chem. Soc. Monograph*, New York, 619 pp.
- RITZMAN, E. G., AND N. F. COLOVOS 1930 Surface areas of sheep. *N. H. Agr. Exp. Sta. Circ.* no. 30, p. 8.
- SCHEUNERT, A., W. KLEIN AND M. STEUBER 1922 Über die Verwertbarkeit des Harnstoffs als Eiweissquelle für Wiederkäuer, zugleich ein Beitrag zur Frage der exkretorischen Funktionen der Haut. *Biochem. Z.*, vol. 133, pp. 137-191.
- SMUTS, D. B. 1935 The relation between the basal metabolism and the endogenous nitrogen metabolism, with particular reference to the estimation of the maintenance requirement of protein. *J. Nutrition*, vol. 9, pp. 403-433.
- SMUTS, D. B., AND J. S. C. MARAIS 1938 The endogenous nitrogen metabolism of sheep with special reference to the maintenance requirement of protein. *Onderstepoort J. Vet. Sci. and An. Indust.*, vol. 11, pp. 131-139.

- SOTOLA, J. 1930 Biological values and supplementary relations of the proteins in alfalfa hay and in corn and sunflower silage. *J. Agr. Res.*, vol. 40, pp. 79-96.
- STUDT, E. 1939 Über die Menge und Verdaulichkeit des durch zymogene Symbiose im Pansen des Wiederkauers erzeugten Bakterieneiweisses auf Grund von Schlachtergebnissen. *Z. f. Tierzüch. u. Züchtungsbiol.*, vol. 44, pp. 253-261.
- WEGNER, M. I., A. N. BOOTH, G. BOHSTEDT AND E. B. HART 1940 The "in vitro" conversion of inorganic nitrogen to protein by micro-organisms from the cow's rumen. *J. Dairy Sci.*, vol. 23, pp. 1123-1129.

# THE VALUE OF UREA IN THE SYNTHESIS OF PROTEIN IN THE PAUNCH OF THE RUMINANT

## II. IN GROWTH<sup>1</sup>

LORIN E. HARRIS AND H. H. MITCHELL

*Division of Animal Nutrition, University of Illinois, Urbana*

(Received for publication February 17, 1941)

In the preceding paper (Harris and Mitchell, '41) it has been shown that urea nitrogen is available to the sheep for the replacement of at least 90% of the endogenous losses of nitrogen, presumably through the mediation of the micro-organisms of the paunch, with an efficiency of about 60%. In the experiment to be reported below, the extent to which urea nitrogen can be used in satisfying the growth requirements of sheep was explored and some information was obtained on the efficiency of utilization.

### PLAN OF THE EXPERIMENT

Twenty-three Montana grade cross-bred wether lambs, ranging in weight initially from 50 to 80 pounds, were the subjects of the experiment. During a preliminary period of 40 days they were fed a ration of corn silage ad libitum, supplemented with limestone, salt and a fortified cod liver oil. The purpose of this period was to determine whether this ration, to be used as a basal ration in the subsequent growth period, was capable of supporting growth or nitrogen equilibrium. Hence, body

<sup>1</sup> The data reported in this paper were taken from a thesis submitted by Lorin E. Harris to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Husbandry, August, 1940.

weights were determined for all lambs at the beginning and end of the period and nitrogen balances measured on three of the lambs.

At the end of the preliminary period, all lambs were sheared and five of them were slaughtered for carcass analysis.

The remaining eighteen lambs were then carried through a growth period of 71 to 111 days. During this period the lambs were fed in six pairs and two trios, for the purpose of comparing the basal ration plus a nitrogen-free supplement (dex-trinized by steam cooking) of starch 83, corn syrup 10, brown sugar 5, salt mixture 1<sup>2</sup> and citric acid 1%, with three rations prepared from the latter by the addition of sufficient urea to raise the protein equivalent ( $N \times 6.25$ ) from an average of 5.35% on the dry basis to approximately 8% in one case, 11% in a second case and 15% in a third.

Three pairs of lambs were used in comparing the 11 and 15% rations, and three other pairs in comparing the 8 and 11% rations. In one trio, the basal ration was compared with the 8 and 11% rations, and in another trio, the basal ration was compared with the 11 and 15% rations. Within all pairs and trios the intakes of silage and of carbohydrate supplements were equalized.

After the first 30 days of feeding, the Ca to P ratio of all rations was adjusted to approximately 2:1, and elemental sulfur was added to all rations in the proportion of one-fifteenth of the urea nitrogen in the 15% protein-equivalent ration.

Nitrogen balances were determined on the lambs during the growth period, using collection periods of 10 days preceded by preliminary periods of 5 days, the food intake being constant for the entire 15 days. At the termination of the growth period, the lambs were slaughtered and their entire carcasses were analyzed for dry matter, nitrogen and gross energy. Initial and final body weights of the lambs were averages of weights taken on 3 consecutive days.

<sup>2</sup>L. G. Wesson, Science, vol. 75, p. 339 (1932).

## THE EXPERIMENTAL RESULTS

*Preliminary period.* During the 40-day preliminary period the ration of corn silage supplemented by minerals and cod liver oil, in the amounts voluntarily consumed, did not maintain the body weight of most of the twenty-three lambs. With the exception of two of the lambs, the food consumption declined during this period. Two of the lambs showed slight increments in body weight of 1.5 and 3.0 pounds, one lamb maintained its weight while all of the rest lost weight, the losses ranging from 0.5 to 10.0 pounds. The average change in weight for the group was -3.1 pounds.

The nitrogen metabolism was studied on this ration with three of the lambs. The results of these studies are summarized in table 1. During the first collection periods the first

TABLE 1

*Nitrogen metabolism of lambs during the preliminary period on the basal ration alone and with a sugar supplement.*

LAMB NO. AND BODY WEIGHT	RATION	DAILY NITROGEN METABOLISM:			
		Intake gm.	Feces gm.	Urine gm.	Balance gm.
2706 60.3 kg.	Basal	4.99	2.61	2.98	-0.60
	Basal + sugar	4.11	3.05	1.67	-0.61
	Basal	4.42	2.65	2.71	-0.94
2837 68.0 kg.	Basal	4.99	2.40	3.01	-0.42
	Basal + sugar	4.11	3.24	1.84	-0.97
	Basal	4.88	2.61	2.58	-0.31
2783 64.5 kg.	Basal + sugar	4.24	4.09	1.44	-1.29
	Basal	4.42	3.56	1.94	-1.08

two lambs consumed 1361 gm. of silage daily. The nitrogen balances were all negative. In order to determine whether these losses of nitrogen were conditioned by an inadequate intake of energy, 125 gm. of glucose were added to the ration of lambs 2706 and 2837 in a second period and in the first collection period for lamb 2783. The intake of silage was somewhat depressed, amounting to 1225 gm. daily for the first two lambs and 1261 gm. for the third, but the energy intake must nevertheless have been greater. The effect of the sugar was to depress the excretion of urinary nitrogen but to increase that of the fecal nitrogen to such an extent that the balances of nitrogen for the first two lambs were even more

negative than in the first collection period. A return to the silage ration in a following collection period reversed these changes.

The digestibility of the nitrogen of the silage was apparently depressed by the sugar supplement even more than would be expected from the resulting increase in the excretion of metabolic nitrogen in the feces. Computations of the biological value of the nitrogen of the silage, using average values, obtained in the preceding studies on maintenance, for the endogenous nitrogen in the urine, per 100 gm. of body weight, and the metabolic nitrogen in the feces per gram of dry matter consumed, gave an average of 59, with individual values of 56, 52, 59, 63 and 64. For the silage and sugar ration, the biological values of the nitrogen were 73, 76 and 79, averaging 76.

The five check lambs slaughtered at the termination of the preliminary period showed an average gastrointestinal fill of 16.6% of the live weight. The average composition of the empty weight was:  $35.53 \pm 0.64\%$  of dry matter,  $2.42 \pm 0.05\%$  of nitrogen and  $2194 \pm 55$  gm. cals. per gram. These average values were used in computing the initial composition of the lambs in the growth experiment.

*Growth period.* With the addition of the carbohydrate supplement and the urea at the start of the growth period, the lambs ate their rations with more relish and the food consumption increased. The period started on December 12, 1937, and, as in the preliminary period, the lambs were housed at the sheep barns. However, on December 27 a spell of sub-zero weather set in and it was impossible to keep the lambs comfortably warm, even with the use of blankets. They were therefore moved to the nutrition laboratory and confined in individual stalls, improvised from wooden hinged barriers, on a concrete floor covered with shavings. During this disturbance the lambs went off feed and their appetites were slow in recovering under laboratory conditions. Hence, for a period of 20 to 40 days the lambs first lost weight and then merely regained their initial weights. From then on, however, the gains were as satisfactory as could be expected from the nature of the rations under test.

In the course of 50 days it became apparent that the lambs in the two trio groups receiving the basal ration alone were not going to gain in weight. Hence, the control lamb in the trio affording a comparison of the 11 and 15% protein rations was given sufficient casein to bring the protein level to 15%, the starch in the carbohydrate supplement being reduced accordingly. Two balance periods were obtained on this trio after this change in feeding was effected.

The control lamb in the other trio also began to refuse feed before a nitrogen balance study could be made. In this case, all three lambs were put upon a ration of alfalfa hay, corn, oats, yeast and mineral supplements for a period of 20 days, after which they were returned to their original rations and management. On the latter regime, their food consumption was such that a nitrogen balance study was carried out, but shortly after this the appetite of the control lamb again declined.

The average daily gains in body weight of these two trios of lambs during the various subperiods into which their experiments were necessarily divided, are collected in table 2. From

TABLE 2  
*Summary by periods of daily gains of lambs in trios 1 and 2.*

TRIO	LAMB NO.	LENGTH OF PERIOD	RATION FED	PROTEIN EQUIVALENT IN RATION	AVERAGE DAILY GAIN
		days		%	pounds
1	2885	50	Basal + urea	14.7	-0.08
	2706	50	Basal + urea	10.9	-0.02
	2850	50	Basal	5.1	-0.19
1	2885	61	Basal + urea	14.2	0.34
	2706	61	Basal + urea	10.6	0.34
	2850	61	Basal + casein	14.4	0.42
2	2754	50	Basal + urea	11.2	-0.11
	2796	50	Basal + urea	8.2	-0.22
	2805	50	Basal	5.8	-0.23
2	2754	20	Normal	16.1	0.65
	2796	20	Normal	16.1	0.78
	2805	20	Normal	16.1	0.75
2	2754	41	Basal + urea	11.2	0.17
	2796	41	Basal + urea	8.2	0.09
	2805	41	Basal	5.8	0.05

these results it is evident, first, that the basal ration alone was unable to support any considerable growth in the amounts consumed, or even to maintain weight, and, second, that the basal ration supplemented with urea was capable of promoting approximately normal gains in body weight.

In confirmation of the latter statement, the three pairs of lambs receiving the rations containing 11 and 15% of protein equivalent made gains during the last 60 days of the experiment averaging 0.26 and 0.25 pound daily, respectively.

The total gains in body weight for the entire growth period (see table 3) reveal a consistent advantage for the 11% ration over the 8% ration among the four possible comparisons. The average difference in total gain was 2.12 kg., the standard deviation of differences, 1.01 kg., and the probability that fortuitous factors would produce an average difference in gain as great or greater than 2.12 kg. is only 0.018 (Student, '08) and hence may be neglected. It may, therefore, be concluded that the additional urea in the 11% ration has induced a greater rate of growth than the 8% ration.

In the four comparisons of the 11 and 15% rations, the average total gains were 8.90 and 7.62 kg., respectively, and the difference between the two averages is clearly insignificant.

The nitrogen metabolism data secured on the lambs during the growth period are summarized in table 4. The three possible comparisons of the rations containing 11 and 8% protein equivalent all favor the higher protein ration in the daily storage of nitrogen, the average difference in nitrogen balance being 1.25 gm. per day. The standard deviation of differences is 0.35 gm. and the probability of a fortuitous result is only 0.018. The nitrogen balance data, therefore, bear out the body weight measurements in indicating a superior performance of the 11% ration over the 8% ration in promoting growth.

The unsupplemented basal ration induced negative nitrogen balances in three of the four tests, and the one positive balance was practically the equivalent of equilibrium, i.e., +0.30 gm. daily.

TABLE 3  
*The total gains in body weight and the total storage of nitrogen and energy for the growing lambs.*

LAMB NO.	PROTEIN EQUIVA- LENT OF FEEDING PERIOD	LENGTH OF FEEDING PERIOD	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	GAIN IN BODY WEIGHT		N CONSUMED		TOTAL STORAGE		BIO- LOGICAL VALUE OF TOTAL N
					%	days	kg.	kg.	pounds	gm.	
Total	Average per day	Total	Total	Total	Total	Total	Total	As urea	Dry matter	Nitrogen	Energy
2909	11	102	28.6	34.0	5.4	0.117	1037	50.4	2762	147.5	22.86
2837	8	102	28.6	33.6	5.0	0.107	762	32.5	1977	86.0	18.90
2783	11	71	26.3	25.6	—0.7	—0.021	592	53.5	271	30.9	—0.17
2776	8	71	26.1	22.9	—3.2	—0.098	406	36.6	448	41.6	2.19
2711	11	71	25.2	30.6	5.4	0.168	835	52.7	2080	110.3	16.02
2692	8	71	24.3	26.9	2.6	0.081	614	36.5	1071	93.4	5.7
2754	11	111	21.8	28.3	6.5	0.130	768	48.1	1651	173.3	10.19
2796	8	111	22.5	26.2	3.7	0.074	573	29.1	1235	86.3	6.69
2805	5	111	23.7	25.2	1.5	0.049	418	0	65	47.8	—2.08
2773	15	110	33.1	41.4	8.3	0.165	2196	64.2	2479	172.8	17.76
2824	11	110	34.0	44.4	10.4	0.202	1631	51.9	4292	243.5	32.69
2830	15	110	24.7	34.2	9.5	0.190	1704	58.4	4331	197.6	31.62
2629	11	110	24.7	33.7	9.0	0.179	1257	50.0	4018	214.1	25.68
2800	15	111	22.7	27.8	5.1	0.101	1317	60.4	2175	70.1	19.49
2701	11	111	21.3	28.6	7.3	0.143	969	46.1	1460	122.7	9.16
2885	15	111	25.6	33.2	7.6	0.151	1548	63.8	1965	199.1	12.57
2706	11	111	25.4	34.3	8.9	0.177	1149	51.3	3366	197.2	24.05

TABLE 4

*Nitrogen metabolism data on growing lambs. Results expressed on the daily basis.*

LAMB NO.	PER CENT PROTEIN EQUIVALENT	NITROGEN SUPPLEMENT	NITROGEN INTAKE		N IN FECES	N IN URINE	N BALANCE	BIOLOGICAL VALUE OF TOTAL N
			Basal ration	Supplement	gm.	gm.	gm.	%
2909	11	Urea	4.90	5.53	3.58	4.10	2.75	67
2837	8	Urea	4.90	2.64	3.35	2.78	1.41	74
2711	11	Urea	6.02	7.51	5.21	4.61	3.71	70
2692	8	Urea	6.02	3.85	5.13	2.65	2.09	79
2754	11	Urea	4.89	4.05	3.18	4.23	1.53	60
2796	8	Urea	4.89	1.72	3.59	2.27	0.75	73
2805	5	None	4.89	0	4.06	1.34	-0.51	63
2805	5	None	4.46	0	3.38	1.48	-0.40	75
2773	15	Urea	5.75	12.39	4.95	11.30	1.89	40
2824	11	Urea	5.75	7.51	4.47	6.26	2.53	60
2830	15	Urea	4.90	9.38	3.21	8.34	2.73	47
2629	11	Urea	4.90	5.53	3.46	4.68	2.29	62
2800	15	Urea	4.92	7.22	2.99	7.61	1.54	41
2701	11	Urea	4.92	3.95	3.13	4.87	0.87	50
2885	15	Urea	4.79	9.12	3.07	7.55	3.29	51
2706	11	Urea	4.79	5.49	3.69	5.30	1.29	53
2850	5	None	4.79	0	3.64	1.45	-0.30	82
2885	15	Urea	5.05	9.38	3.65	8.17	2.61	47
2706	11	Urea	5.05	5.53	3.39	5.05	2.14	59
2850	5	None	5.05	0	3.24	1.51	0.30	83
2885	15	Urea	5.11	8.94	3.98	7.40	2.67	51
2706	11	Urea	5.11	5.25	3.32	4.17	2.87	68
2850	15	Casein	5.07	8.98	3.48	6.80	3.77	57
2885	15	Urea	6.36	9.71	4.39	8.20	3.50	53
2706	11	Urea	6.36	5.60	3.65	5.16	3.15	65
2850	15	Casein	6.32	9.75	4.65	6.93	4.49	61

Comparing the 15 and 11% rations, only five of the seven pairings favor the former ration. The average daily balances of nitrogen were, respectively, 2.60 and 2.16 gm. daily, and the average difference, 0.44 gm., is statistically insignificant ( $s = 0.76$  and  $P = 0.11$ ). Thus, again the balance data support the growth data, in this case indicating no significant difference in growth-promoting properties between the 15 and the 11% rations.

The conclusion may, therefore, be drawn from the latter comparison, either that the ration providing 11% protein equivalent satisfies completely the nitrogen requirement of these lambs for maintenance and growth, or that the upper limit in the rate of protein synthesis from urea in the paunch was reached on the 11% ration. The former conclusion is not out of harmony with unpublished experimental results from this laboratory indicating that the growth requirements of lambs of approximately the size of those used in this experiment can be completely satisfied by a ration containing not much more than 9% of protein (Mitchell and Hamilton, '31-'32, '35-'36).

The two tests with casein indicate a clear superiority of casein nitrogen at the level of 15% protein equivalent over the urea nitrogen at either the 15 or the 11% protein equivalent level. If these differences were statistically significant, the conclusion would be justified that the rate of protein synthesis from urea in the paunch is not sufficiently rapid under the conditions of this experiment to provide fully for the protein demands for growth.

It is interesting to note that the digestibility of dry matter was distinctly less for the lambs on the basal ration than for the lambs on the urea-containing and casein-containing rations, averaging 62.2, 73.3 and 71.3%, respectively.

The storages of nitrogen and energy throughout the growth period of all lambs slaughtered and analyzed, estimated from the chemical composition of the entire carcasses and from the average composition of the check lambs sacrificed and analyzed at the beginning of the experiment, are summarized in table 3. Lamb 2850 was not slaughtered, because the change from the basal ration to a casein-containing ration would render ambiguous the results of a carcass analysis. Lamb 2837 died of acute indigestion about 10 days before it was planned to terminate the feeding period, an event bearing no apparent relation to the type of ration consumed. Its pair mate, 2909, was slaughtered at this time and both carcasses were analyzed. Because of the poor food consumption of lambs 2776 and 2692,

both receiving the 8% ration, the feeding period of the corresponding pairs was terminated earlier than planned.

The average compositions of the lambs in the various groups are summarized in table 5.

TABLE 5

*The average chemical composition of the lambs on the various experimental rations.*

APPROXIMATE PROTEIN EQUIVALENT OF RATION	NUMBER OF LAMBS IN GROUP	COMPOSITION OF EMPTY CARCASSES		
		Dry matter	Nitrogen	Gross energy
%		%	%	cal. per gm.
11	4	37.55	2.59	2378
8	4	37.98	2.62	2419
5.8	1	35.41	2.61	2057
15	4	38.26	2.50	2494
11	4	38.50	2.53	2459

These values reveal nothing abnormal in the composition of the carcasses of the lambs receiving the urea rations, and no marked differences in composition produced by the different levels of urea fed.

Among the four possible comparisons of the storage of nitrogen on the 11 and 8% rations, three indicated greater storages on the 11% ration. The one exception relates to the pair of lambs, nos. 2783 and 2776, that had the poorest appetites throughout the growth period and failed even to maintain their initial weight. These conditions are not conducive to an accurate estimate, which must be by difference, of the change in composition of the carcass of experimental animals by the slaughter method. Hence, it seems fair to minimize the importance of this apparent exception to the rule illustrated by the remaining three comparisons. The carcass analysis data, therefore, may be considered as not out of harmony with the growth data and the nitrogen balance data in indicating a superior growth-promoting value of the 11% ration over the 8% ration.

On the other hand, the nitrogen storages secured on the 15 and 11% rations reveal no significant differences, in con-

sonance with the growth data and the nitrogen metabolism data.

The biological values of the total dietary nitrogen were computed both from the balance data (table 4) and from the slaughter data (table 3). In both cases the basal dietary nitrogen is necessarily included in the calculations, as well as the urea nitrogen and the casein nitrogen. Average values for the excretion of endogenous nitrogen in the urine (0.033 gm. per kilogram body weight) and of metabolic nitrogen in the feces (0.56 gm. per 100 gm. of dry matter consumed), obtained in the preceding experiment on the maintenance lambs, were assumed in making these calculations. The calculations from the balance data were made by the method usually employed in this laboratory (Mitchell, '24), while those made from the slaughter data were necessarily different though they possess the same significance. In the latter case, the total nitrogen storage plus the estimates of total body losses in urine and feces (endogenous + metabolic nitrogen) was divided by an estimate of absorbed nitrogen obtained by multiplying the total nitrogen intake for each lamb by the true digestibility of nitrogen computed from the results of the nitrogen balance studies.

The average biological values for the nitrogen of the basal ration, at a level of 5 to 6%, were 81 from the balance data (four cases) and 100 from the slaughter data on one lamb. For the rations containing 8% protein equivalent (34% of the nitrogen in the form of urea), the average biological values were 75 from the balance data and 74 from the slaughter data. For the rations containing 11% protein equivalent (50% of the nitrogen derived from urea), the average biological values were 63 from the balance data and 58 from the slaughter data. For the rations containing 15% protein equivalent (62% of the nitrogen derived from urea), the average biological values were 47, as obtained from the balance data, and 41, as obtained from the slaughter data. The latter values may be compared with an average biological value of 59 for the 15% casein ration obtained from the balance data on lamb 2850.

In accordance with general experience, the biological values of the urea rations decrease as the concentration of conventional protein increases, from 74 at the 8% level, to 60 at the 11% level, and to 44 at the 15% level. The values obtained from the two sets of data agree remarkably well considering that the balance data were obtained over periods of 10 days and the slaughter data over periods of 71 to 111 days.

For urea alone, Fingerling and associates ('37) have computed the efficiency of utilization on two growing calves to be 50 and 61%, using nitrogen balance data on a low-protein ration and on the same ration plus a urea supplement. The latter ration did not support maximum retention of nitrogen. These values are comparable with those obtained in this experiment for the total nitrogen of the 11% ration.

The average standard deviations of the biological values about their respective group means were 5.0, for those derived from the balance data, and 3.2 for those derived from the slaughter data. Mitchell, Burroughs and Beadles ('36) have reported an average standard deviation of 3.7 for an extensive series of biological values, each of which was secured with a group of nine or ten rats.

The 15, 11 and 8% protein equivalent rations contained, respectively, 3.16, 1.88, and 0.93% of urea on the dry basis on the average. In order to detect evidence of possible renal damage, the kidneys of all slaughtered lambs were weighed and examined histologically by Dr. C. C. Morrill of the Division of Animal Pathology and Hygiene. In the four possible comparisons of the 15 and 11% rations, the kidney weight was consistently greater on the higher level of urea, averaging for both kidneys, 92.5 gm. for the 15% lambs and 80.0 gm. for the 11% lambs. However, between the lambs on the 11 and the 8% rations no consistent difference in kidney weight was observed, the average weights being 68.8 and 72.5 gm., respectively.

The histological examination revealed no changes in structure that are not often seen in animals receiving normal

rations. Although the urine volumes on all levels of urea were measured in a few cases, no evidence of diuresis was obtained. It seems fair to conclude that, in the concentrations fed and under the conditions of these experiments, urea did not exert an appreciable toxic effect.

#### CONCLUSIONS

1. The addition of urea to a low-nitrogen ration that is in itself unable to support appreciable growth in lambs or even consistently to maintain nitrogen equilibrium, converts it into a ration capable of promoting a normal or nearly normal rate of growth. Such a ration need contain no more than 11% of conventional protein ( $N \times 6.25$ ), in which urea provides 50% of the nitrogen.
2. The biological value of the nitrogen of a ration of silage and a carbohydrate supplement, containing 5.35% of conventional protein on the dry basis, is about 82. With successive additions of urea to produce rations containing approximately 8, 11 and 15% of protein equivalent, the average biological values are 74, 60 and 44, respectively.
3. Rations of the type used in this experiment, containing up to 3.16% urea on the dry basis, do not exert any observable toxic effect on lambs over a feeding period of 110 days. At the highest level, some renal hypertrophy results, but there is no histological evidence of kidney damage.

The authors wish to acknowledge the invaluable advice and assistance of Dr. W. G. Kammlade and Mr. W. J. Hampton of the Sheep Husbandry Division in the feeding and care of the lambs employed in these experiments.

#### LITERATURE CITED

- FINGERLING, G., B. HIENTZSCH, H. KUNZE AND K. REIFGERST 1937 Ersatz des Nahrungseiweißes durch Harnstoff beim wachsenden Rinde. Landw. Vers.-Stat., vol. 128, pp. 221-235.
- MITCHELL, H. H. 1924 A method of determining the biological value of protein. *J. Biol. Chem.*, vol. 58, pp. 873-903.

- MITCHELL, H. H., E. W. BURROUGHS AND J. R. BEADLES 1936 The significance and accuracy of biological values of proteins computed from nitrogen metabolism data. *J. Nutrition*, vol. 11, pp. 257-274.
- MITCHELL, H. H., AND T. S. HAMILTON 1931-1932 Current feeding standards for lambs can be improved. 45th Ann. Rpt. Ill. Agr. Exp. Sta., pp. 95-97.
- \_\_\_\_\_ 1935-1936 Lambs require less protein than commonly supposed. 49th Ann. Rpt. Ill. Agr. Exp. Sta., pp. 104-106.
- STUDENT 1908 The probable error of a mean. *Biometrika*, vol. 6, pp. 1-25.

## THE EFFECT OF HEAT ON THE AVAILABILITY OF THE IRON OF BEEF MUSCLE

HELEN G. OLDHAM

*Department of Pediatrics, The University of Chicago, Illinois*

TWO FIGURES

(Received for publication January 31, 1941)

In a previous paper (Oldham, Schlutz and Morse, '37) we reported that, as judged by the amount of iron retained, a normal infant was able to utilize the iron of cooked beef muscle equally as well as that of an iron salt. This finding was surprising, since Sherman, Elvehjem and Hart ('34) had reported that less hemoglobin was formed in anemic rats fed beef muscle than in those fed the same amount of iron in the form of iron salts.

In the present study we have attempted to find an explanation for the difference between our results and those of the Wisconsin group by comparing the hemoglobin levels of anemic rats after feeding equal amounts of iron as ferric chloride and as beef muscle. The beef muscle was fed both before and after heating in order to determine whether or not the process of heating affected the utilization of the iron of the meat. The study was divided into three parts in each of which matched pairs of animals received equivalent amounts of iron in different forms. In the first part ferric chloride and oven-dried meat were fed; in the second part, ferric chloride and vacuum-dried meat; in the third, oven-dried meat and vacuum-dried meat.

## EXPERIMENTAL

*Treatment of animals*

Young male rats from mothers who had received our stock ration were weaned at 18 days of age. They were then housed in glass cages as described elsewhere (Oldham and Schlutz, '40) and fed a diet of fresh whole milk. Approximately 6 weeks were required for depletion of iron reserves and during the last 2 weeks of this period copper and manganese salts were added to the milk diet to insure complete depletion. When the hemoglobin levels were reduced to 3.0–4.0 gm. per 100 ml. of blood, the animals were paired as to litter, hemoglobin level and weight. Supplementary feedings were then begun and were continued through a period of 6 weeks.

*Diets*

The animals received a basal diet of milk and copper and manganese salts in addition to their supplementary feedings of either ferric chloride or meat. Fresh whole unpasteurized milk was obtained from a local dairy. Amounts fed daily were measured by pipettes into the feeding cups. A solution of copper sulphate and manganese chloride equivalent to 0.05 mg. of copper and 0.04 mg. of manganese was added to each milk cup before feeding. The supplements of ferric chloride were prepared in the laboratory from iron wire. They were measured by pipette and mixed with a small amount of milk. All supplements were given in the morning and were entirely consumed before additional food was offered. The meat was purchased as round steak, ground and dried either in an oven at 80°C. or under vacuum at room temperature. After it was dried, it was ground again in a mortar and the daily portions were weighed on an analytical balance.

In the first experiment, one group of animals received daily 68 ml. of milk and 2.0 ml. of ferric chloride ( $\approx$  0.196 mg. Fe). Their litter mates received 40 ml. of milk, 2.57 gm. of oven-dried meat and 1 gm. of cane sugar. The cane sugar was sub-

stituted for its caloric equivalent of milk to reduce the protein content of the diet and thus make it more comparable to that of the animals receiving ferric chloride supplements. The milk in the diet of both groups of animals was increased gradually as the experiment progressed, by amounts which insured good gains. The amount of milk given was always such that it was completely consumed by all animals. It was considered essential to keep the diets isocaloric in order that the weight gains of the two groups of animals would be comparable, since the hemoglobin level on a given iron intake is affected by the total blood volume and this in turn is proportional to the body weight.

The animals in experiments 2 and 3 were treated similarly to those in experiment 1 with the exception of the supplements fed. In experiment 2 one group of animals received ferric chloride, their litter mates, vacuum-dried meat. In experiment 3 one group was fed oven-dried meat, the other vacuum-dried meat.

#### *Methods of analysis*

Samples of milk were analyzed for iron and the results obtained were used throughout the study. Each lot of meat was analyzed separately and an amount fed which kept the daily iron intake constant. The ferric chloride solutions were also analyzed for iron. The amyl alcohol thiocyanate method was used for all iron determinations.

Hemoglobin determinations were made at the beginning of each experiment and at the end of the second, fourth, fifth and sixth weeks. The method used was that of Evelyn ('36).

#### DISCUSSION OF RESULTS

The daily iron intakes and the average gain in hemoglobin of animals on the different supplements are shown in table 1. The average hemoglobin levels on the various supplements are also shown in figure 1. When the chief source of iron was oven-dried meat, the average hemoglobin gain was slightly,

TABLE 1

*Daily iron intake and hemoglobin formation of animals receiving different supplements*

EXP.	NO. OF ANIMALS	SUPPLEMENT TO MILK DIET	DAILY INTAKE OF Fe	AV. Hb. LEVEL AT BEGINNING	AV. Hb. LEVEL AT END	AV. GAIN IN Hb.
			mg.	gm./100 cc.	gm./100 cc.	gm./100 cc.
1	6	Oven-dried meat	0.257	3.35(2.98-3.75)	12.39(10.14-14.30)	9.04(6.59-11.16)
	6	Fe Cl <sub>3</sub>	0.257	3.26(2.88-3.70)	11.12(10.06-13.18)	7.86(6.03- 9.48)
2	5	Vacuum-dried meat	0.260	3.68(2.98-4.07)	9.47( 8.88-10.22)	5.79(5.54- 6.26)
	4	Fe Cl <sub>3</sub>	0.261	3.60(2.98-4.02)	13.17(12.17-13.72)	9.57(9.19-10.12)
3	5	Oven-dried meat	0.264	3.56(3.14-3.86)	13.26(12.09-14.30)	9.70(8.70-10.44)
	4	Vacuum-dried meat	0.259	3.54(2.78-4.07)	7.68( 7.45- 7.85)	4.14(3.76- 4.67)
Averages:		Oven-dried meat	0.260	3.45	12.79	9.34
		Fe Cl <sub>3</sub>	0.259	3.40	12.18	8.78
		Vacuum-dried meat	0.260	3.62	8.67	4.61

but not significantly greater than that found when an equal amount of iron was fed as ferric chloride (9.3 gm. and 8.8 gm. respectively per 100 ml. of blood). When, however, the chief source of iron was meat which had not been heated, the average hemoglobin level was significantly less than that for the other two groups, even though the total daily iron intake was the same for all three groups.

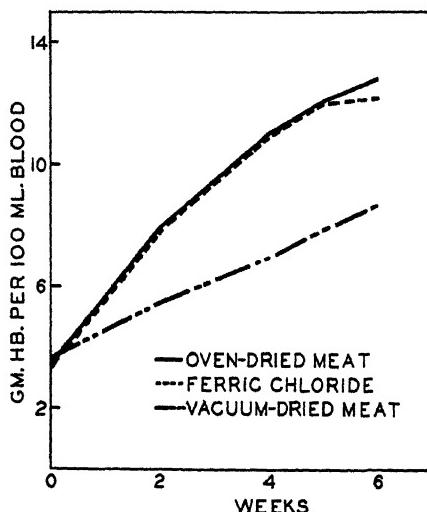


Fig. 1 Average hemoglobin formation on different supplements.

The same results are observed when individual hemoglobin values of paired animals are compared. It is evident (fig. 2) that, in each pair of animals, although slight variations occurred, the one which received oven-dried meat or ferric chloride was able to build almost twice as much hemoglobin as the litter mate who received vacuum-dried meat. It may also be noted that when one of a pair received oven-dried

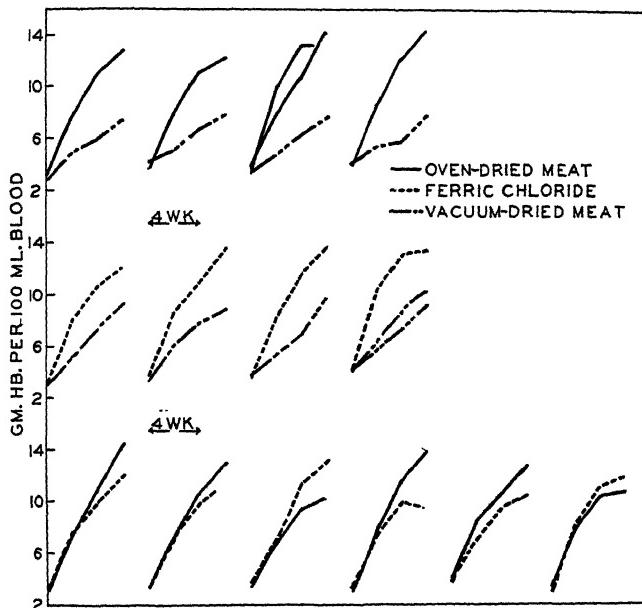


Fig. 2 Hemoglobin formation of individual animals.

meat and the other ferric chloride, the hemoglobin gains were, in every case, almost identical.

There seems to be little doubt but that at least 50% of the iron of beef is in the organic form, and is incorporated in the hemoglobin molecule. In a compilation of results from various laboratories McCance ('39) states that values ranging from 50 to 85% have been reported as representing the proportion of iron which did not react with dipyridyl reagent. The findings of Shenk, Hall and King ('34) substantiate this amount.

After analyzing the ribeye muscle of beef for muscle hemoglobin and blood hemoglobin they reported values of 0.332 to 0.454% (for the former) and 0.015 to 0.041% (for the latter).

The amount of dried meat given daily in the present study was 2.5 gm. which represented approximately 9 gm. of fresh meat. This amount of meat would contain 31 to 46 mg. of hemoglobin, if it is assumed that the percentages of muscle hemoglobin and blood hemoglobin in the meat used in this study were the same as those reported by Shenk and his co-workers. This would mean that 0.11 to 0.16 mg. of the iron intake of the animals receiving meat supplements was organic (of hemoglobin origin) since the iron content of both forms of hemoglobin according to Millikan ('39) is the same (0.345%). As the total iron content of the beef fed was approximately 0.24 mg., and according to the above calculations 0.11 to 0.16 mg. was organic, it would seem justifiable to consider at least 50% of the iron of beef to be in the organic form.

It seems therefore, that the effect of the heat was to render the organic iron of meat as available as that of an inorganic iron salt. In light of this observation, the results of our previous study seem reasonable, since the infant was fed meat which had been cooked.

The manner in which heat affects this change in the availability of iron is an open question. That heat renders the beef more digestible seems improbable, since the animals receiving the two types of meat made comparable weight gains. A possible explanation might be that heat so affects the hemoglobin molecule as to make it more easily broken down and thus more readily assimilated by the body.

#### SUMMARY

Young rats made anemic by a milk diet were given supplements of ferric chloride, oven-dried meat and vacuum-dried meat.

When equal amounts of iron were fed in the form of the above-named supplements approximately the same amount of hemoglobin formation was observed when the supplement was

oven-dried meat as when it was ferric chloride. Significantly less hemoglobin formation occurred when vacuum-dried meat was fed.

Heat renders the iron of beef muscle of which at least 50% is in the organic form, as available for hemoglobin synthesis as the iron of an inorganic iron salt.

#### LITERATURE CITED

- EVELYN, K. A. 1936 A stabilized photoelectric colorimeter with light filters. *J. Biol. Chem.*, vol. 115, p. 63.
- MCCANCE, R. A. 1939 The ionizable and available iron in food. *Chem. and Industry*, vol. 58, p. 528.
- MILLIKAN, G. A. 1939 Muscle Hemoglobin. *Physiol. Rev.*, vol. 19, p. 503.
- OLDHAM, H., F. W. SCHLUTZ AND M. MORSE 1937 Utilization of organic and inorganic iron by the normal infant. *Am. J. Dis. Child.*, vol. 54, p. 252.
- OLDHAM, H., AND F. W. SCHLUTZ 1940 The effect of different levels of vitamin B<sub>1</sub> and iron on the retention of iron and the fat content of normal young rats. *J. Nutrition*, vol. 19, p. 569.
- SHENK, J. H., J. L. HALL AND H. H. KING 1934 Spectrophotometric characteristics of hemoglobins. *J. Biol. Chem.*, vol. 105, p. 741.
- SHERMAN, W. C., C. A. ELVEHJEM AND E. B. HART 1934 Further studies on the availability of iron in biological materials. *J. Biol. Chem.*, vol. 107, p. 383.



# CARBOHYDRATE STORAGE AND MOBILIZATION IN THE RAT

M. MASON GUEST<sup>1</sup>

*Department of Physiology, College of Physicians and Surgeons, Columbia University*

FIVE FIGURES

(Received for publication February 25, 1941)

## I. GENERAL STANDARDIZATION OF BIOLOGICAL AND TECHNICAL PROCEDURES IN GLYCOGEN DETERMINATIONS

A striking feature of the published data on liver and muscle glycogen is the variability in concentration found among animals which were presumably under the same experimental conditions. As a result numerous animals must be used before safe conclusions can be drawn, even though the experimental changes are large. Thus, it is all but impossible to draw conclusions when the effect of the experimental procedure falls within the range of the usual control fluctuation, even with exceptionally large samples.

The results of a number of recent investigators, summarized in table 1, emphasize the variability in reported glycogen concentrations. Not only are wide deviations encountered from laboratory to laboratory, but in many cases in the results of a given author.

It appeared to be essential, therefore, to increase the biological, i.e., animal to animal, precision of the determination before entering upon a research in which the expected glycogen changes were of ordinary physiological magnitude. As a measure of the difference between animals the coefficient of dispersion, calculated from  $\varepsilon$ , has been used  $\epsilon = \sqrt{\frac{\sum d^2}{N-1}}$ . The

<sup>1</sup> Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science, Department of Physiology, Columbia.

TABLE 1  
*Liver and muscle glycogen-control values.*

INVESTIGATOR	NO. OF RATS	AGE OR WEIGHT	PREVIOUS DIET	TERMINAL PROCEDURES	LIVER		MUSCLE	
					Mean	C.D.	Mean	C.D.
24-hour fasted rats								
		days (d.) grams (g.)						
Barbour et al., '27	24 ♂, ♀	110-136 g.	Balanced	A	0.16	37.5	0.30	23.3
Blatherwick et al., '35	15 ♂	70-150 d.	Balanced	B	0.56	89.0	0.66	120.0
Blatherwick et al., '35	14 ♀	70-150 d.	Balanced	B	0.21	219.0	0.61	34.4
Buell et al., '36	9 ♂	100-150 g.	Balanced	B	0.34	....	0.46	....
Catron and Lewis, '29	16 -	100-150 g.	Balanced	C	0.09	54.4	....	....
Cori and Cori, '28 a, b	4 ♂	123-141 g.	Balanced	B	0.17	29.4	....	....
Cori, '32	15 ♂	.....	.....	B	....	....	0.53	10.0
Deuel et al., '34	10 ♂	"Adult"	Glucose	B	1.54	....	0.39	....
Deuel et al., '34	10 ♀	"Adult"	Glucose	B	0.93	....	0.40	....
Evans, '34	13 ♂, ♀	150-200 g.	Balanced	D	0.19	73.0 <sup>1</sup>	0.52	7.7 <sup>1</sup>
Hynd and Rotter, '30	....	63- 84 d.	Balanced	E	0.9	....	0.51	....
Hynd and Rotter, '30	....	63- 84 d.	Cheese	E	0.68	....	0.29	....
Karczag et al., '25	24 ♂, ♀	126-275 g.	Balanced	A	0.16	56.2	0.27	11.1
Sahyun et al., '34	9 ♂	165-235 d.	77.5% CHO	D	....	....	0.45	8.9
Sahyun et al., '34	10 ♂	165-235 d.	54.0% CHO	D	....	....	0.28	29.0
Fed rats								
Barbour et al., '27	7 ♂, ♀	159-400 g.	Balanced	A	2.10	19.5	0.34	14.7
Britton and Silvette, '32	6 ♂, ♀	30- 60 g.	Balanced	-	1.05	16.2	0.47	10.6
Collip et al., '36	11 ♂, ♀	60- 90 d.	Balanced	B	....	....	0.53	15.1
Cori and Cori, '33	5 -	.....	.....	B	....	....	0.64	15.0
Deuel et al., '34	10 ♂	"Adult"	High CHO	B	3.77	....	0.43	....
Deuel et al., '34	10 ♀	"Adult"	High CHO	B	3.74	....	0.40	....
Deuel et al., '37	10 ♂	73- 76 d.	Balanced	B	4.25	14.4	....	....
Deuel et al., '37	10 ♀	73- 76 d.	Balanced	B	2.59	27.6	....	....
Evans, '34	6 -	.....	.....	D	2.08	33.3 <sup>1</sup>	0.64	5.7 <sup>1</sup>
Greisheimer and Johnson, '29	♂, ♀	42 d.	45% sucrose	F	3.01	....	0.31	....
Greisheimer and Johnson, '30 b	30 ♂, ♀	40- 44 d.	Balanced	F	3.34	26.5 <sup>2</sup>	0.30	21.4 <sup>2</sup>
Greisheimer and Johnson, '30 b	33 ♂, ♀	40- 44 d.	60% starch	F	3.74	24.8 <sup>2</sup>	0.31	27.2 <sup>2</sup>
Greisheimer and Johnson, '30 b	30 ♂, ♀	40- 44 d.	60% sucrose	F	4.43	21.8 <sup>2</sup>	0.29	27.7 <sup>2</sup>
Greisheimer and Johnson, '30 b	30 ♂, ♀	40- 44 d.	60% lard	F	3.85	20.9 <sup>2</sup>	0.31	26.0 <sup>2</sup>
Greisheimer and Johnson, '30 b	30 ♂, ♀	40- 44 d.	60% casein	F	2.68	31.5 <sup>2</sup>	0.33	24.4 <sup>2</sup>
Hynd and Rotter, '30	- ♂, ♀	63- 84 d.	Balanced	E	3.1	....	0.59	....
Hynd and Rotter, '30	- ♂, ♀	63- 84 d.	Cheese	E	1.85	....	0.56	....

Terminal procedures—A = decapitation, B = amyta, C = chloroform, D = nembutal, E = stunned and decapitated, F = stunned and spinal section. C.D. = coefficient of dispersion.

<sup>1</sup>The deviations given were assumed to be the deviation of the mean ( $\epsilon_M$ ).

<sup>2</sup>The deviations given were assumed to be the probable error of the mean.

deviation usually given,  $\epsilon_M$ , is a measure of the precision of the mean (Scott, '27). Since the present interest is in the precision of individual values this has been calculated to  $\epsilon$  when necessary in the preceding summary.

Although no absolute measure of accuracy is available, larger glycogen recoveries are probably an indication that the analysis has more closely approximated the actual tissue concentration. On the basis of this criterion an improvement in accuracy also has been accomplished.

The final procedure is actually a synthesis and coordination of the published contributions of many individual workers besides ourselves.

### *Methods*

Considerable care has been exercised in the selection and preparation of the subjects. The reported results include only determinations on 100-day-old, male albino rats of the Sherman strain.

The Rockland dry pellet rat diet<sup>2</sup> (which contains 50% carbohydrate, 25% protein and 5% fat) has been used. All fed rats have been subjected to a 48-hour inanition period preceding the final 12 hours of feeding. This insures that the animals eat uniformly at the selected time. To simulate the natural nocturnal feeding conditions the animal room was lighted until the food was provided and was kept dark until the last animal had been removed for sampling.

The amount of food that had passed the stomach during the final feeding period was determined in the following manner. The rats, in individual cages, were allowed food ad libitum during the allotted period. After removal of the tissues for analysis, the stomach contents were dried over a steam bath and the weight deducted. Figure 1 indicates the relation between the amount of food per gram of body weight which passed the stomach and the liver glycogen concentration. By means of such a curve it is possible to adjust all liver glycogen

<sup>2</sup> Manufactured by the Arcady Farms Milling Company, Chicago, Ill.

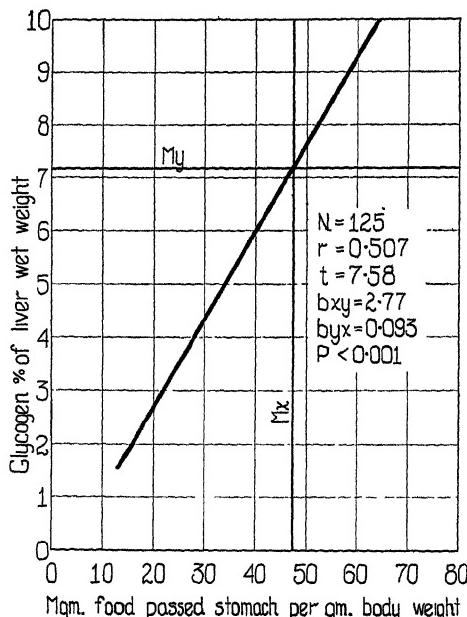


Fig. 1 Relationship between the food which passed the stomach and the percentage of liver glycogen. The heavy  $r$  line corresponds to the least square criterion for the plot of the individual points. The regression lines are not shown, but may be plotted from the constants given.

Explanation of symbols:

$N$  = the number of experimental animals.

$M_x$  and  $M_y$  are the means of the  $x$  and  $y$  values respectively.

$r$ , the correlation coefficient =  $\Sigma xy / \sqrt{\Sigma x^2 \times \Sigma y^2}$ . This may take any value between  $-1$  and  $+1$ . If  $r$  is zero the two factors are independent, while the nearer  $r$  approaches  $\pm 1$ , the greater the degree of correlation. The deviation of  $r$  ( $\sqrt{\frac{1-r^2}{N-1}}$ ) is significant only for large samples ( $>100$ ) and with a normal distribution of  $r$ .

$t$  = test of significance of the correlation coefficient in terms of the standard error. Given  $t$  and  $N$ , the probability ( $P$ ) may be obtained from Fisher's table.

The regression equations may be expressed as  $X = M_x + b_{xy} (y - M_y)$  and  $Y = M_y + b_{yx} (X - M_x)$  where  $X$  is the average value of  $x$  for a given value of  $y$ ,  $M_x$  is the mean of the  $x$ -values and  $b_{xy}$  is the regression coefficient of  $x$  on  $y$ .

concentrations to a standard amount of food entering the intestine. It was found, however, that in the reported series the elimination of all animals having had less than 40 mg. of food per gram of body weight passed the stomach during the feeding period gave essentially the same glycogen precision as by adjustment. On this basis less than 5% of all animals have been rejected.

The effects of small temperature fluctuations are uncertain, but rats kept at 12°C. for 12 to 14 hours prior to sampling had 45% less liver glycogen and 12% less muscle glycogen than similar animals at 28°. We therefore, like Barbour et al. ('27), maintained the animals at  $28 \pm 1^\circ$  during the preliminary procedure.

Nembutal has been used as the anesthetic. The advantages of a short induction period are obvious and the high glycogen concentrations, "normal" blood sugar and lactic acid<sup>3</sup> concentrations which we have obtained are indicative that glycogenolysis with this anesthetic has been minimal.

The technic of administration of the anesthetic has been found to be important. Less excitation occurred if the rat's head was covered with a small towel while still in its cage, and kept covered until after injection. With 80 mg. of nembutal per kilogram of body weight, intraperitoneally, the animal passes into a state of deep anesthesia within 2 to 3 minutes.

The gastrocnemius muscles have been frozen *in situ*. We have been unable to show any significant difference between the two gastrocnemii of the same animal when both are frozen *in situ*, yet in ten experiments when one was so frozen and the other muscle carefully dissected out and then frozen immediately, the glycogen concentration was significantly lower ( $0.87 \pm 0.02$ ) than that of the contralateral muscle ( $1.09 \pm 0.02$ ).

<sup>3</sup> Although lactic acid determinations were not carried out on all animals, the results obtained indicate that a minimal change has occurred during the terminal procedures. Thus a group of twelve fed rats gave the following mean values: muscle glycogen,  $0.93 \pm 0.02\%$ ; blood sugar,  $104 \pm 3$  mg. per cent and lactic acid,  $19.5 \pm 1.6$  mg. per cent.

In addition, maintenance of the tissue in the frozen state prior to the action of the alkali retards enzymatic action.

In liver and muscle glycogen determinations any extraneous tissue constituent tends to decrease both the accuracy and precision of measurement (Guest, '38). Fenn ('39) has recently shown that the blood content of the liver may be reduced as much as 5% by a severe hemorrhage prior to its removal. Consequently 4 to 5 cc. of blood were drawn as rapidly as possible by heart puncture; the abdomen was quickly opened by longitudinal incision, and the liver was immediately removed and packed in CO<sub>2</sub> snow. The subcutaneous fat which might be included in the muscle sample was quickly stripped away after the leg had been skinned prior to being chilled.

The frozen tissue was crushed to a powder by the method described by Graeser et al. ('34). The crushed tissues are placed on waxed paper alternately with CO<sub>2</sub> snow. In this way a number of tissue samples may be crushed in sequence and preserved frozen for mixing and introduction into the KOH. The connective tissue was separated from the muscle by shifting the powdered tissue back and forth a few times on the waxed paper. The tissue was weighed in KOH just above the dew point. This avoids loss by evaporation and gives no indication of glycogen loss.

The Blatherwick et al. ('35) modification of the Good, Kramer and Somogyi method ('33) has been used, further modified as noted. After the acid hydrolysis, glucose was determined by the method of Shaffer and Hartmann ('21), with use of the table and the precautions given by Duggan and Scott ('26). The reducing action of non-glycogen substances does not seem to be significantly great. This was shown by adding the supernatant fluid from centrifuged saliva to the crushed tissue and incubation at 40°C. for 30 minutes in a pH 6.9 buffered solution. Less than 0.05% reducing substance per gram of wet tissue was recovered when the digested tissue was carried through the glycogen method.

*Results*

In a series of sixty-seven rats, standardized as described, a mean liver glycogen (as glucose) of  $8.25 \pm 0.79$  gm. per 100 gm. of wet tissue and a mean muscle glycogen of  $1.06 \pm 0.13$  gm. per 100 gm. of wet tissue were obtained. The coefficients of dispersion were 9.6 and 12.3 respectively.

When these results are compared with those obtained by other workers (see table 1) the increase in the level and the decrease in the coefficients of dispersion are apparent. In another series of twenty rats in which (1) the temperature, (2) the light, (3) the food intake, (4) excitation and (5) the hemorrhage were uncontrolled the mean glycogen values were respectively  $5.6 \pm 1.2$  and  $0.81 \pm 0.33$  for liver and muscle. The differences in both recovery and precision between the two groups are obvious.

## II. NUTRITIONAL CONTROL AS A MEANS FOR THE STANDARDIZATION OF LIVER AND MUSCLE GLYCOGEN CONCENTRATIONS AT DESIRED LEVELS

The possibility of experimental modification in tissue glycogen concentrations is frequently dependent on the control levels. Controlled variation of the nutritional state appears to be the most physiological and direct means of establishing these control concentrations at a convenient level. Procedures are described which have been found capable of varying the liver glycogen concentration from practically zero to over 8% of the tissue wet weight. In this work certain additional facts were brought out which are of interest.

*Methods*

In addition to the Rockland rat diet (designated "carbohydrate"), two others have been used: One, a protein diet, consisting of meat scraps,<sup>4</sup> contained 55% protein, 5.6% fat, and less than 1% carbohydrate. The other, designed to promote gut activity while furnishing little or no caloric value,

<sup>4</sup> Armour's.

contained agar-agar (100 gm.), mineral oil (4 gm.) and Liebig's extract (1 gm.); these ingredients were thoroughly mixed by grinding.

All animals were fed the Rockland rat diet until 7 days before the tissue analysis, and some until the preliminary fasting periods. During the 7 days preceding sampling the protein diet animals were allowed to eat the meat scraps *ad libitum* except during the preparatory fasting periods. Animals fed on both diets were fasted for 48 hours as a preliminary to the final 12-hour feeding. The agar-fed animals received the Rockland rat diet followed by 24 hours of agar-agar feeding. In each experimental group carbohydrate and protein pre-fed rats were fasted at atmospheric pressure and served as controls for animals in the same nutritional states which were fasted at one-half atmosphere pressure as described by Evans ('34).

### *Results*

The mean values are summarized in table 2. The total blood sugar and liver and muscle glycogen are shown in figure 2. In this figure the carbohydrate-fed group is considered as 100%, and the values for each of the other groups are given in relation to this.

### *Discussion*

By means of the feeding procedures that have been described, it is possible to obtain liver glycogen concentrations of less than 0.1% to over 8% of the liver wet weight. The muscle glycogen concentrations exhibit less variation between the groups, the lowest being 0.60% for the agar-fed rats and the highest 1.06% for the carbohydrate-fed group.

The effect of the proportion of protein upon the glycogen concentrations of the tissues is dependent upon whether the analysis immediately follows feeding or 24 hours of inanition. Carbohydrate-fed rats yielded over three times as much liver glycogen and a significantly greater muscle glycogen con-

centration than those similarly fed the protein diet. Our results thus confirm the conclusions of Greisheimer and Johnson ('30 a) relative to liver glycogen and give evidence of a related muscle glycogen behavior.

In contrast to those fully fed, animals pre-fed with meat scraps and fasted for 24 hours invariably exhibited a higher liver glycogen concentration than those pre-fed with the carbohydrate diet. The probability of this difference is high when both groups are maintained at atmospheric pressure.

TABLE 2  
*The tissue glycogen and blood sugar in various nutritional conditions.*

GROUP NO.	DIET	INANITION	NO. OF RATS	MEAN LIVER GLYCOGEN AND PRECISION OF MEAN	$\sqrt{\frac{\sum d^2}{N-1}}$	MEAN MUSCLE GLYCOGEN AND PRECISION OF MEAN	$\sqrt{\frac{\sum d^2}{N-1}}$	MEAN BLOOD SUGAR AND PRECISION OF MEAN	$\sqrt{\frac{\sum d^2}{N-1}}$
1	CHO	None	67	8.25±0.10	±0.79	1.06±0.02	±0.13	104±2	±15
2	Prot.	None	8	2.52±0.18	±0.52	0.81±0.02	±0.06	105±4	±12
3	CHO	24 hr.	10	0.35±0.09	±0.30	0.68±0.02	±0.06	91±6	±18
4	Prot.	24 hr.	10	1.01±0.07	±0.23	0.70±0.02	±0.06	110±3	±11
5 <sup>1</sup>	CHO	24 hr.	10	1.77±0.18	±0.56	0.85±0.04	±0.12	123±4	±13
6 <sup>1</sup>	Prot.	24 hr.	10	3.74±0.25	±0.79	0.96±0.05	±0.17	129±4	±13
7	Agar	A	13	0.08±0.01	±0.05	0.60±0.01	±0.05	104±2	± 9
8	CHO	B	8	6.24±0.20	±0.55			102±2	± 7
9	CHO	C	5	5.91±0.23	±0.48			103±2	± 4

<sup>1</sup> Maintained at one-half atmosphere during the inanition period.

A = Agar-agar fed during the final 24-hour period.

B = Allowed 5.1 gm. Rockland rat diet per 100 gm. rat during the final 12-hour feeding period.

C = Allowed 4.0 gm. Rockland rat diet per 100 gm. rat during the final 12-hour feeding period.

Glycogen given as glucose in per cent of tissue wet weight.

Blood sugar given in milligrams per cent.

The same relation, but with greater difference, is found when the rats are subjected to a pressure of one-half atmosphere during the fasting period. Differences in muscle glycogen and blood sugar between the protein and carbohydrate groups are not statistically significant, but seem to parallel that of the liver glycogen.

These results are not in agreement with the conclusions of most other workers. MacKay and Bergman ('33), working with three rats per group, reported a higher glycogen concentration in the livers of carbohydrate pre-fed animals after 24 hours of starvation than in those pre-fed on a diet predominately protein or fat. However, after 48 hours of fasting their rats which were pre-fed with the high protein diet showed higher liver glycogen than did those pre-fed with a high carbohydrate diet. Bollman and Mann ('36) also reported similar results with dogs, but did not state the number of animals used nor the experimental deviations. The workers cited fed their experimental diets from 18 to 45 days. Markedly unbalanced diets, when fed over a long period of time, may conceivably alter the liver function.

Our results thus indicate that an adequate supply of carbohydrate maintains the liver glycogen at a higher level during feeding than a diet deficient in carbohydrate, but high in protein. In contrast, 24-hour fasted animals are better able

---

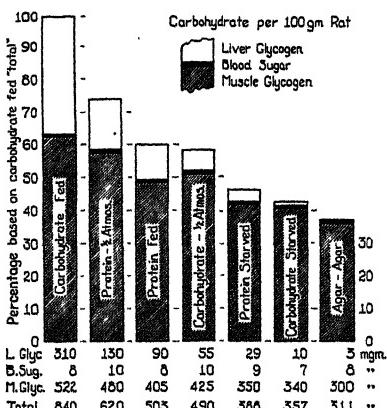
Fig. 2 Representation of the distribution of liver and muscle glycogen and blood sugar in the experimental groups. The calculation of the total liver glycogen is based on the weight of the liver and the animal and the determined glycogen percentage. The muscle glycogen value is based on the assumption that the muscle tissue makes up 50% of the body weight and that the flexor leg muscles are representative of the muscles as a whole. The total sugar in the blood is calculated from the blood sugar determination and the assumption that the blood constitutes 8% of the body weight.

Fig. 3 The relation between the blood sugar and the log. value of the liver glycogen concentration. In all fed groups studied the mean blood sugar is approximately 104 mg. per cent, regardless of the liver glycogen concentration. The r line for the fasted groups and the locations of the means are indicated. For the forty fasted animals,  $r = 0.770$ ,  $t = 6.67$ ,  $P = 0.001$ ,  $b_{xy} = 0.002$  and  $b_{yx} = 29.16$ . The results of a few other investigators are also shown. Because of lack of space on the graph the reference Cori, '28 a, '28 b, refers to Cori and Cori, '28 a, '28 b; MacKay, '33, to MacKay and Bergman, '33, and Lawrence, '31, to Lawrence and McCance, '31.

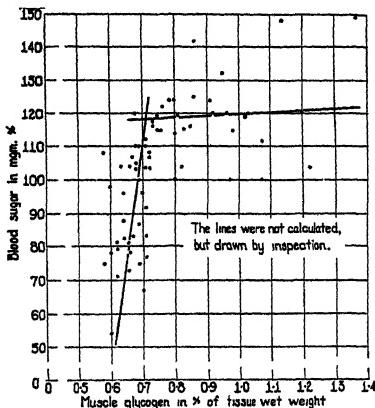
Fig. 4 Graphical representation of the relation between the muscle glycogen and blood sugar concentrations in fasted animals.

Fig. 5 The relation between muscle and liver glycogen in fasted and in fed animals. The upper curves have been recalculated on a total liver and muscle glycogen basis as explained in the text. The lower curves give the relations between the concentrations.

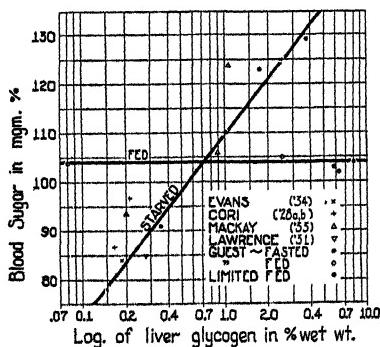
to maintain the liver glycogen when pre-fed a high protein diet. For protection during periods of inanition, a high protein diet for a comparatively short period previous to the fast, is indicated.



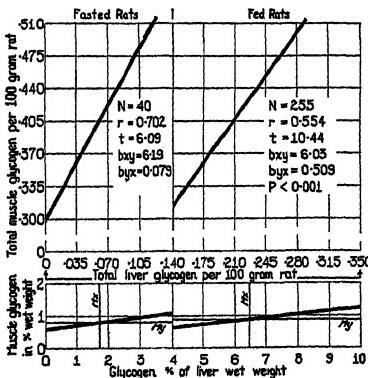
2



4



3



5

The character of the metabolic changes which occur during fasting may become more apparent through an experimental treatment which exaggerates or amplifies these changes. Evans ('34, '36) reported that when he kept rats at a pressure of one-half atmosphere during the period of fasting, the liver glycogen was sensibly the same as that of comparable fully-

fed animals, whereas the nitrogen excretion was higher. Evans's data together with our own results provide strong evidence that gluconeogenesis from protein is accelerated under the low pressure conditions and that protein is made more readily available for gluconeogenesis during periods of fasting by means of protein excess in the diet previous to inanition.

The effect of an active gut in fasted animals was investigated by means of the agar-agar diet. Although the animals on this diet were without absorbable food for the same length of time as were the 24-hour fasted animals, both the liver and muscle glycogen concentrations were found to be significantly lower than with any of the fasted groups. It is worth noting that three animals which were given magnesium sulfate by stomach tube and then fasted for 24 hours showed liver and muscle glycogen concentrations (0.15 and 0.64) comparable to the agar animals. Several interpretations, not mutually exclusive, of these low values are possible: (1) Increased gut activity may deplete the glycogen stores through increased energy demands. (2) The agar-agar acting as a mild cathartic may empty the gut of absorbable materials more effectively than fasting. (3) The active gut may call forth circulatory, hormonal or nervous responses which influence glycogenesis or glycogenolysis in the liver.

### III. THE INTERRELATIONSHIPS OF THE CONCENTRATIONS OF TISSUE CARBOHYDRATES

These were studied by a regression analysis (Fisher, '36) of the data accumulated in part II and representing about 300 animals. In all cases the fasted and fed animals were treated separately.

1. *Blood sugar-liver glycogen.* No demonstrable relationship has been found between the blood sugar and the liver glycogen in any of the fed groups. The mean blood sugars in these groups show no significant differences, regardless of the liver glycogen concentration. In figure 3 the mean values

for all of the fed groups fall on or near a line parallel to the x-axis.

In contrast to the results in the fed groups, the mean blood sugar concentrations for the fasted groups vary directly as a function of the log. value of the liver glycogen concentration (fig. 3). The correlation coefficient ( $r$ ) for the fasted individuals is 0.770 and  $t$  is 6.67. Thus the probability of an important physiological relation is indicated; the physiological significance being measured by  $r$  and the probability by  $t$ .

The conclusion that different mechanisms are responsible for the blood glucose-liver glycogen relationship in feeding and in fasting animals is not warranted by the evidence available. However, it does appear that physiological adjustments are made in the transition between the two nutritional states. It may be significant that Cori, Cori and Schmidt ('39) in phosphorylation studies obtained more potent phosphorylase extracts from the livers of well-fed rabbits than from those of fasting animals.

2. *Blood sugar-muscle glycogen.* Although no relationship could be found between the blood sugar and muscle glycogen concentrations of the fed animals, in the fasted rats there appears to be a definite tendency for the individual muscle glycogen-blood sugar values to fall about the curve indicated in figure 4. This type of curve is not susceptible to treatment by ordinary regression methods, as there appears to be an inflection at a muscle glycogen concentration of about 0.75% and a blood sugar concentration of about 120 mg. per cent. Thus the relationships between the blood sugar and the muscle glycogen appear to be made up of two components. Below the inflection the muscle glycogen decreases gradually while the blood sugar falls rapidly, indicating the maintenance of a minimal muscle glycogen, even though the blood sugar values are low. Above the inflection the blood sugar, although showing considerable variation, exhibits no general tendency toward higher concentrations as the muscle glycogen increases to 1.40%. This appears to indicate that the muscles are capable of acting as carbohydrate reservoirs when the blood sugar

concentration rises above a physiological level. It is evident that such a mechanism would be effective in conserving carbohydrate and in preventing the blood sugar from reaching the renal threshold.

3. *Liver-muscle glycogen.* The correlation between the liver and muscle glycogen concentrations for both the fed and the fasted rats is high. The calculated straight lines which best represent the distribution of the individuals are shown in the lower parts of figure 5.

The principal difference in the curves representing the two nutritional states is that, although they are roughly parallel, the straight-line relationship of the fasted group is displaced upward on the muscle glycogen axis. Thus a greater percentage of muscle glycogen is found in the fasted animals per unit percentage of liver glycogen than in the fed rats. This displacement may be a reflection of decreased muscular activity in the fasted rats or it may be indicative of a generally altered metabolism with redistribution of the carbohydrate stores. Both of these factors are possibly involved.

In contrast with the large differences between the extremes of liver and muscle glycogen concentrations, the total liver glycogen, when plotted against the total muscle glycogen, exhibits a more nearly one to one proportionality. The latter relationships are shown in the upper part of figure 5. In estimating the total muscle glycogen, the skeletal muscles were assumed to constitute 50% of the body weight and the glycogen percentage in the flexor leg muscles was assumed to be representative of the muscles as a whole. The total liver glycogen value is based on the actual wet weight of the liver and the determined glycogen concentration. These have been recalculated on the basis of a 100-gm. rat. Although the total muscle glycogen is about ten times that of the total liver glycogen, they tend to increase or decrease together. In the economy of the organism, therefore, the greater mass of muscle tissue appears to be a major factor in sustaining its relatively constant glycogen concentration.

## SUMMARY

1. By means of the described procedures the recovery of glycogen from the liver and muscle of fed rats is higher than has been previously reported with a substantial reduction in the coefficient of dispersion.

2. (a) By means of nutritional control, liver glycogen concentrations may be established to a practical precision at any desired level (from 0 to 8% of the tissue, wet weight). (b) Rats on a diet predominating in carbohydrate have a significantly higher liver glycogen concentration at the end of the feeding period than rats on a diet high in protein. However, the reverse is true after the food has been withheld for 24 hours. (c) Maintenance of the animals at a pressure of one-half atmosphere during the fasting period exaggerates the effects of the pre-inanition diets on the liver and muscle glycogen and the blood sugar concentrations.

3. (a) It has been indicated by a regression analysis that a positive physiological relation exists between the blood sugar and the log. value of the liver glycogen concentration in fasted rats and between the liver and muscle glycogen concentrations in both fed and fasted rats. (b) Indications are given of a relationship between the blood sugar and the muscle glycogen concentrations in fasted rats.

The author wishes to express his sincere thanks and deep appreciation to Dr. E. L. Scott for his invaluable guidance and assistance; to Dr. J. J. McBride for his aid and suggestions; to Dr. W. S. Root for suggestions in the preparation of the manuscript; and to Miss Ruth A. Rawson for her technical assistance.

## LITERATURE CITED

- BARBOUR, A. D., I. L. CHAIKOFF, J. J. R. MACLEOD AND M. D. ORR 1927 Influence of insulin on liver and muscle glycogen in the rat under varying nutritional conditions. *Am. J. Physiol.*, vol. 80, p. 243.
- BLATHERWICK, N. R., P. J. BRADSHAW, M. E. EWING, H. W. LARSON AND S. D. SAWYER 1935 The determination of tissue carbohydrate. *J. Biol. Chem.*, vol. 111, p. 537.

- BOLLMAN, J. L., AND F. C. MANN 1936 The physiology of the impaired liver. *Ergebn. d. Physiol.*, vol. 38, p. 445.
- BRITTON, S. W., AND H. SILVETTE 1932 Effects of cortico-adrenal extract on carbohydrate metabolism in normal animals. *Am. J. Physiol.*, vol. 100, p. 693.
- BUELL, M. V., I. A. ANDERSON AND M. B. STRAUSS 1936 On carbohydrate metabolism in adrenalectomized animals. *Am. J. Physiol.*, vol. 116, p. 274.
- CATRON, L. F., AND H. B. LEWIS 1929 The formation of glycogen in the liver of the young white rat after the oral administration of glycerol. *J. Biol. Chem.*, vol. 84, p. 553.
- COLLIP, J. P., D. L. THOMSON AND G. TOBY 1936 The effect of adrenaline on muscle glycogen in adrenalectomized, thyroidectomized and hypophysectomized rats. *J. Physiol.*, vol. 88, p. 191.
- CORI, C. F., AND G. T. CORI 1928a The fate of sugar in the animal body. VIII. The influence of insulin on the utilization of glucose, fructose and dihydroxyacetone. *J. Biol. Chem.*, vol. 76, p. 755.
- 1928b The mechanism of epinephrine action. II. The influence of epinephrine and insulin on the carbohydrate metabolism of rats in the postabsorptive state. *J. Biol. Chem.*, vol. 79, p. 321.
- 1933 A comparison of total carbohydrate and glycogen content of mammalian muscle. *J. Biol. Chem.*, vol. 100, p. 323.
- CORI, G. T. 1932 Carbohydrate changes during anaerobiosis of mammalian muscle. *J. Biol. Chem.*, vol. 96, p. 259.
- CORI, G. T., C. F. CORI AND G. SCHMIDT 1939 The role of glucose-1-phosphate in the formation of blood sugar and synthesis of glycogen in the liver. *J. Biol. Chem.*, vol. 129, p. 629.
- DEUEL, H. J., JR., M. GULICK, C. F. GRUNEWALD AND C. H. CUTLER 1934 The sexual variation in carbohydrate metabolism. III. The comparative glycogen and fat content of the liver and muscles of rats and guinea pigs. *J. Biol. Chem.*, vol. 104, p. 519.
- DEUEL, H. J., JR., J. S. BUTTS, L. F. HALLMAN, S. MURRAY AND H. BLUNDEN 1937 The sexual variation in carbohydrate metabolism. IX. The effect of age on the sex difference in the content of liver glycogen. *J. Biol. Chem.*, vol. 119, p. 617.
- DUGGAN, W. F., AND E. L. SCOTT 1926 A critical examination of four methods commonly used for the determination of sugar in the blood. *J. Biol. Chem.*, vol. 67, p. 287.
- EVANS, G. 1934 The effect of low atmospheric pressure on the glycogen content of the rat. *Am. J. Physiol.*, vol. 110, p. 273.
- 1936 The adrenal cortex and endogenous carbohydrate formation. *Am. J. Physiol.*, vol. 114, p. 297.
- FENN, W. O. 1939 The deposition of potassium and phosphate with glycogen in rat livers. *J. Biol. Chem.*, vol. 128, p. 297.
- FISHER, R. A. 1936 Statistical methods for Research Workers, 6th ed., Oliver and Boyd.

- GOOD, C. A., H. KRAMER AND M. SOMOGYI 1933 The determination of glycogen. *J. Biol. Chem.*, vol. 100, p. 485.
- GRAESER, J. B., J. E. GINSBERG AND T. E. FRIEDEMANN 1934 A method for the analysis of tissues. *J. Biol. Chem.*, vol. 104, p. 149.
- GREISHEIMER, E. M., AND O. H. JOHNSON 1929 Glycogen formation in rats. *Am. J. Physiol.*, vol. 90, p. 369.
- 1930 a Glycogen formation in rats. *Am. J. Physiol.*, vol. 93, p. 653.
- 1930 b Glycogen formation in rats. I. Diets containing about sixty per cent of the total caloric value in the form of starch, sucrose, lard and casein. *Am. J. Physiol.*, vol. 94, p. 11.
- GUEST, M. M. 1938 On the determination of glycogen in muscle. *J. Biol. Chem.*, vol. 123, p. xlvi.
- HYND, A., AND D. L. ROTTNER 1930 Studies on the metabolism of animals on a carbohydrate-free diet. I. The distribution of glycogen and fat in the liver of animals fed on a carbohydrate-free diet. *Biochem. J.*, vol. 24, p. 1390.
- KARCZAG, L., J. J. R. MACLEOD AND M. D. ORR 1925 The use of the albino rat in insulin standardization. *Trans. Roy. Soc. Can.*, vol. 19, p. 57.
- LAWRENCE, R. D., AND R. A. McCANCE 1931 The effect of starvation, phloridzin, thyroid, adrenaline, insulin and pituitrin on the distribution of glycogen in the rat. *Biochem. J.*, vol. 25, p. 570.
- MACKAY, E. M., AND H. C. BERGMAN 1933 The influence of the preceding diet upon the rate of glucose absorption and glycogen synthesis. *J. Nutrition*, vol. 6, p. 515.
- SAHYUN, M., R. SIMMONDS AND H. WORKING 1934 The effect of diet on the distribution of glycogen in the skeletal muscle of the rat. *Am. J. Physiol.*, vol. 108, p. 708.
- SCOTT, E. L. 1927 What constitutes an adequate series of physiological observations? *J. Biol. Chem.*, vol. 73, p. 81.
- SHAFFER, P. A., AND A. F. HARTMANN 1921 The iodometric determination of copper and its use in sugar analysis. II. Methods for the determination of reducing sugars in blood, urine, milk and other solutions. *J. Biol. Chem.*, vol. 45, p. 365.



# HISTOLOGICAL STUDIES OF THE TISSUES OF RATS FED A DIET EXTREMELY LOW IN ZINC<sup>1</sup>

RICHARD H. FOLLIS, JR., HARRY G. DAY<sup>2</sup> AND E. V. McCOLLUM

*Department of Pathology, School of Medicine, and Department of Biochemistry,  
School of Hygiene and Public Health, The Johns Hopkins University,  
Baltimore, Maryland*

TWO PLATES (ELEVEN FIGURES)

(Received for publication March 10, 1941)

Investigation at the University of Wisconsin (Todd, Elvehjem and Hart, '34; Stirn, Elvehjem and Hart, '35; Hove, Elvehjem and Hart, '37, '38) and recent studies in our laboratory (Day and McCollum, '40) have conclusively demonstrated the indispensability of zinc in the nutrition of the rat. The only pathological change that has been reported is a loss of hair about the neck and shoulders extending, sometimes, to involve the entire ventral surface of the body (Todd, Elvehjem and Hart, '34).

The present report deals with a detailed microscopic study of the tissues of rats which were the subject of a preliminary report (Day and McCollum, '40) on the preparation of a diet very low in zinc content, and observations on the growth, appearance, and phosphatase activity of blood, bone and kidney, as well as the carbonic anhydrase activity of blood.

## MATERIALS AND METHODS

Histological observations were made on fourteen rats from three litters (table 1). The mothers of two litters were

<sup>1</sup> Aided by a grant from the Rockefeller Foundation to Dr. E. V. McCollum.

<sup>2</sup> Present address: Department of Chemistry, Indiana University, Bloomington, Indiana.

restricted to the zinc-deficient diet when the young were 12 days old. The third litter was taken from the stock colony when the young were about 25 days old. The rats were kept on monel metal screens in pyrex glass jars. Pyrex glass rods, supported by wooden frames, were used to cover the jars. Precautions were taken to keep dust contamination at a minimum.

The preparation of the diet has been given elsewhere (Day and McCollum, '40). Seven rats were given the deficient diet and supplements. The total intake of zinc was not more than 2 to 4  $\mu\text{g}$ . of zinc per rat daily. Seven littermate controls received the same diet and supplements plus 0.15 mg. Zn (as  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) in 0.2 ml. of water per rat daily. The diet was given ad libitum. The daily supplement was composed of liver concentrate (equivalent to 6.4 gm. liver), 40  $\mu\text{g}$ . of thiamine and 1.0 mg. of choline. Water was redistilled in pyrex glass.

The rats were killed with chloroform and immediately autopsied at the times shown by table 1. The tissues were fixed in Zenker-Formol solution, imbedded in paraffin, and stained with haematoxylin and eosin. Other special stains were used as indicated.

#### GROSS EFFECTS OF THE DEFICIENCY

Table 1 indicates the survival period of each animal. Three zinc-deficient rats died; two of litter no. 2 dying after 33 and 34 days respectively, and one of litter 3 dying after 61 days on the diet. Control animals were killed and autopsied at those times, as indicated in the table.

As shown in table 1, growth became greatly retarded in the Zn-deficient rats after the first 2 or 3 weeks. After 3 to 5 weeks, depending somewhat on the degree of deficiency as indicated by growth, the hair became sparser in all of the Zn-deficient rats, but at this time there were no completely denuded areas. Practically all of the subcutaneous fat disappeared early. After approximately 70 days Zn-deficient rats nos. 2 and 6 had developed definite areas of

alopecia. In no. 2, two denuded areas occurred in the mid-dorsal region, one measuring about  $3.5 \times 2.0$  cm. and the other about  $2.5 \times 1.0$  cm. In no. 6, the denuded area was over the right shoulder. The denuded spaces of nos. 2 and 6 were roughened and scaly. Apparently these areas were painful. A few days before the animals were killed they began to chew the afflicted parts, causing them to bleed.

TABLE 1

*Effect of dietary zinc deficiency on the growth and survival of rats*

	RAT AND LITTER NUMBER	SEX	DAYS ON DIET <sup>1</sup>	WEIGHT IN GRAMS							
				Initial	2 wks.	4 wks.	6 wks.	8 wks.	9 wks.	10 wks.	11 wks.
Zinc deficient	1L3	M.	61 (d)	54	77	84	95	105	96	77	78
	2L3	M.	74 (k)	40	55	51	67	75	76		
	5L1	M.	50 (k)	37	51	65	70				
	6L1	Fe.	74 (k)	34	53	59	74	78	84	88	90
	7L1	M.	74 (k)	37	58	79	69	102	101	107	110
	10L2 <sup>2</sup>	M.	33 (d)	23	41	40					
	11L2	Fe.	34 (d)	25	39	42					
	12L2	M.	34 (k)	28	42	48					
Average weight				35	52	59	75	90	89	91	93
Zinc added	3L3	M.	74 (k)	38	64	100	154	180	189	200	207
	4L3	M.	61 (k)	46	81	125	179	223	234		
	8L1	Fe.	74 (k)	32	50	85	128	152	160	168	180
	9L1	M.	50 (k)	35	61	99	146				
	13L2	Fe.	74 (k)	24	55	77	98	121	131	140	155
	14L2	M.	33 (k)	24	62	89					
	15L2	M.	74 (k)	28	69	102	162	196	217	226	231
Average weight				32	63	97	145	174	186	184	193

<sup>1</sup> K—killed; D—died.

<sup>2</sup> Animal died during the night. Tissues were not used for histological studies.

slightly. Number 7 rat developed alopecia over the lower back but dermatitis did not occur. Thus all three of the Zn-deficient rats that lived more than 61 days developed macroscopic skin lesions. The tails appeared normal. Considerable amounts of hair were found in the stomachs of all the Zn-deficient animals.

About 18 days before the experiment was ended one control female, no. 13, was placed in the cage of a control male.

When the female was autopsied six fetuses were found, giving additional evidence that the experimental diet was adequate except for zinc.

#### MICROSCOPICAL FINDINGS

*Alimentary system.* Since the oesophagus showed the most pronounced change this will be described first. The buccal lesions were less marked. The normal lining of the oesophagus consists of a basal layer of cells in which occasional mitotic figures are seen. On this are several layers with larger clearer nuclei whose cell borders are indistinct. There is an abrupt transition to a keratinized layer on which are usually adherent bacteria (figs. 1 and 3). In contrast to this the Zn-deficient animals showed a different picture. The cells of the basal layer were more numerous and closely packed but there were no more mitotic figures than in the controls. In the overlying stratum the cells were also more numerous and instead of being two or three layers thick, they averaged six or eight cells thick. The nuclei were a little smaller and the nuclear membrane was wrinkled. No mitotic figures were found in these cells. On the inner edge of this layer the nuclei became smaller and stained deeper but did not disappear as occurs normally. Instead there was a stratum consisting of small dark nuclei four or five layers deep imbedded in a pink-staining, homogeneous material. There was slight keratinization of the inner margin. There were large numbers of gram positive cocci adherent to the edge (figs. 2 and 4). The epithelium of the posterior roof of the mouth showed the same change though not to so marked a degree; no change was found in the anterior portion. In occasional foci the tongue exhibited the alteration too. The marked change in the oesophagus began sharply at the base of the tongue and extended throughout the entire length to its entrance into the fore stomach, at which point it ceased. The two portions of the stomach were normal as were the small and large intestines. The salivary glands, liver, and pancreas showed no change. The latter organ was stained

to bring out the cytology of the islet cells but no differences were noted.

*Circulatory system.* Sections of the heart and blood vessels from the Zn-deficient rats did not differ from their controls.

*Respiratory system.* The mucosa of the nares of the deficient animals was entirely normal as were the glands beneath. The trachea was lined by ciliated epithelium; the bronchi and pulmonary parenchyma were entirely normal.

*Genito-urinary system.* Normal spermatogenesis was taking place in the one male animal autopsied at 50 days. However, in rats autopsied after 61 and 74 days on the diet, although sperm was being formed in some of the tubules, there were numerous atrophic ones lined only by spermatogonia. Some contained giant cells such as are seen in chronic inanition. All the tubules were smaller than those of the controls. The epithelium of the accessory glands was not so columnar and these structures were smaller in the deficient rats.

In the ovaries, corpora lutea were not found in the deficient rats. The follicles had partially developed, however. The epithelial cells lining the uterus were lower and non-vacuolated; there were only a few glands as compared with those of the controls. One normal (no. 13) showed decidual tissue. The vagina of the deficient rats showed keratinized epithelium with leucocytes.

The kidneys, their pelvis, the ureters and bladder were normal in all of the deficient animals.

*Nervous system.* Sections of the brain showed no noteworthy differences between the deficient animals and their controls.

*Myeloid and lymphoid systems.* The marrow of the long bones of the deficient animals showed no changes from the normal. The lymphoid tissue in the deficient animals was less conspicuous in the Zn-low animals. All spleens and thymus glands were smaller in the deficient animals than in their controls.

*Skeleton.* Sections of the upper tibiae revealed differences between the two groups. The epiphyseal cartilage was a little

narrower in the deficient animals and the hypertrophic cells were not as numerous. The trabeculae at the cartilage-shaft junction were thinner and shorter in the Zn-low animals than in their controls. There seemed to be fewer osteoblasts in the former group.

*Muscle.* No alterations in the smooth or striated muscle of the deficient animals could be found.

*Ductless glands.* The thyroid glands of the deficient rats tended to have smaller follicles but no other changes could be detected. The parathyroid glands of the Zn-deficient rats were not different from the controls. The adrenals of the deficient and control animals were alike. The hypophyses of the deficient animals were definitely smaller than those of the controls. Sections were stained to bring out the chromophilic granules. The number of basophile cells was about the same in both groups. The eosinophilic cells showed no very great difference in the two groups either.

*Skin.* No changes occurred in the skin of the control animals. Definite lesions were found in five out of the seven Zn-deficient rats (figs. 6-11). The earliest change which was found at 33 days consisted of a hyper-keratinization. Coincident with this there was a thickening of the epidermis. Instead of having a normal thickness of three to four cell layers, the epithelium increased to eight to ten cells in thickness. The epithelium lining the hair follicles became keratinized as well. As the epidermis increased in thickness, there was no appreciable increase in mitotic figures in the basal layer; however, mitoses could be detected in the cells lying above. Another prominent feature was the presence of clear spaces in the cytoplasm of the basal cells as well as those above and the nuclei became more pyknotic. The cells, too, became spread apart by fluid since intercellular spaces were noted. At this stage there was a crust lying on the epidermis consisting of keratinized fibers, bacteria and leucocytes. In the corium there was little or no oedema; but there were in later stages leucocytes, both polymorphonuclear as well as monocytes. In the later stages, too, corresponding with the

areas from which the hair had been lost, changes appeared in the follicles and these disappeared completely leaving only a few mononuclear cells to mark where they had been. The sebaceous glands, however, remained and the individual cells were larger. In none of the animals was there a disappearance of these structures. As noted grossly, this change took place over the dorsum of the animals. Similar microscopic changes about the nose were found in four out of seven deficient animals. No lesions were found on the plantar surface nor were there any changes in the ears.

*Eyes.* Lesions of the cornea were noted in two of the seven deficient animals (nos. 5 and 6). In the tunica propria there was a dense cellular infiltration consisting of mononuclear and polymorphonuclear leucocytes, with the former predominating. They were found throughout but were more numerous just beneath the epithelium. Blood vessels were also present (fig. 5). Descemet's membrane was intact. There was vesiculation of some of the basal epithelial cells and in places the epithelial cells were spread apart. There was no increased keratinization of the cornea or conjunctivae. No changes were found in the lacrimal glands.

#### DISCUSSION

We have presented a detailed histological study of the tissues of rats restricted to a dietary intake of 2 to 4  $\mu\text{g}$ . Zn per rat daily. Changes were found in the oesophagus in all the animals and to a lesser extent in the mouth. Lesions were noted in the skin in five of the seven deficient animals and changes in the eyes in two of the seven Zn-deficient rats.

In the oesophagus, there was a thickening of the epithelial cell layer together with the presence of an inner layer of incompletely keratinized cells. No evidence of an increased proliferation of cells could be found as evidenced by mitotic figures. The changes can be interpreted as due either to a retardation in the normal keratinization of the epithelium which then is not removed or to an increased proliferation of cells. The change is identical with the parakeratosis which

one encounters in human skin lesions such as psoriasis. These changes were seen to a slighter degree in the buccal cavity. The epithelium lining the fore stomach was not involved. Such an alteration of the oesophagus in rats is unique. It does not resemble the lesions of vitamin A deficiency (Wolbach and Howe, '25) which does not involve the oesophagus nor did the deficient animals show changes associated with this deficiency in the other tissues.

The alterations in the skin consisted of a hyperkeratinization, thickening of the epidermis, intra- and intercellular oedema and loss of hair follicles with preservation of the sebaceous glands. Secondary bacterial invasion took place later. It is to be emphasized that the plantae and ears showed nothing. Skin lesions were found in five of the seven Zn-deficient rats.

Todd, Elvehjem and Hart ('34) noted a loss of hair on the ventral surfaces of the rats in which was demonstrated the indispensability of the Zn in the diet. In contrast, the alopecia and changes in the skin were most marked over the dorsum of our animals.

Changes in the skin of rats have been noted in a wide variety of deficiency states (Sullivan and Nicholls, '40 a). Until recently, however, accurate histological studies using purified diets lacking one or more known components have not been undertaken. The changes that have been described in the Zn-deficient animals have no counterpart, to our knowledge, in any of the other nutritional dermatoses in the rat. Sullivan and Nicholls ('40 b) have described the lesions in vitamin B<sub>6</sub> deficiency which consist of hyperkeratosis, acanthosis and oedema of the corium with leucocytic infiltration over the paws, nose, chin, submental region and upper chest. The sebaceous glands and hair follicles remain intact. In riboflavin deficiency these authors ('41) found partial alopecia over the venter with inflammation of the eyelids and spectacle alopecia in some animals. Microscopically, there is a disintegration of the sebaceous glands and disappearance of the hair follicles with atrophy of the epidermis. The change

associated with vitamin H deficiency (Gyorgy, Sullivan and Karsner, '37), or egg-white injury, consists of acanthosis with inter- and intra-cellular oedema, hyperkeratosis and atrophy of the hair follicles and sebaceous glands. From the present state of our knowledge, it seems safe to conclude that Zn-deficiency produces a definite pathological picture in the skin of the rat.

As noted above, corneal lesions were found in two of the seven deficient animals. This change consisted of vascularization and leucocytic infiltration. The picture seems similar to that recently described by Bessey and Wolbach ('39) in uncomplicated riboflavin deficiency in rats. These authors noted extreme vascularization followed later by leucocytic infiltration. They were unable to produce the change by other means, such as fasting, old age, vitamin B<sub>2</sub> and B<sub>6</sub> deficiency and it disappeared when riboflavin was added to the diet.<sup>3</sup> The same vascular proliferation and cellular infiltration have been noted in vitamin A deficiency by Wolbach and Howe ('25). It must be pointed out that since the corneal epithelium showed no evidence of characteristic keratinization this deficiency may be dismissed. In our present state of information and since the change occurred in so few animals, we do not feel justified in drawing any conclusions as to the effect of Zn-deficiency on the cornea.

As noted above, changes were found in the gonads and accessory sexual organs. The atrophy of the testicular tubules and partial lack of spermatogenesis together with the failure of the ovarian follicles to mature were interpreted to be due to inanition and we did not feel they were produced directly by Zn-deficiency. The changes in the secondary sexual organs were those characteristic of retarded development and were doubtless also associated with partial inanition.

The skeleton showed a slowing of growth and the histological picture was a non-specific one such as is seen in athreptic

<sup>3</sup>In a recent personal communication Dr. Wolbach writes, "the one preparation of the cornea that you sent seems to be very much like the riboflavin-deficiency corneas".

animals. This was interesting since chemical analyses showed that the average concentration of Zn in the femurs of the deficient rats was only 94.7  $\mu\text{g}$ . per gram of ash as compared with 236.6  $\mu\text{g}$ . per gram of ash in the controls.

Since Zn has been shown to be an important constituent of insulin, the islets of Langerhans in the pancreas were studied with interest. No changes could be detected, however. It may be pointed out that Hove, Elvehjem and Hart ('38) found no disorder of carbohydrate metabolism in the animals they placed on a low Zn diet. These workers postulated that there was a decreased absorption of foodstuffs through the intestinal mucosa.

The activity of intestinal phosphatase (Hove, Elvehjem and Hart, '40 a), blood phosphatase (Day and McCollum, '40) and carbonic anhydrase (Hove, Elvehjem and Hart, '40 b) is decreased by a deficiency of zinc. Also, the absorption of protein is impaired by zinc deficiency (Hove, Elvehjem and Hart, '38). Since riboflavin is essential in the metabolism of amino acids, as a flavin-adenine-dinucleotide (Theorell, '40), it seems permissible, in view of the findings reported here, to hypothesize that a deficiency of zinc might affect the respiratory functions of the flavoproteins. In view of this the possibility of a relationship between zinc and the metabolism of riboflavin is being investigated by one of us (H. G. D.).

#### SUMMARY

In rats fed a diet adequate in all known respects except extremely low in zinc content, specific pathological changes were noted in the oesophagus, and to a lesser extent in the buccal cavity and the skin, and in a few animals, in the cornea.

The oesophagus showed extreme parakeratosis with a thick layer of partially keratinized cells.

The skin showed hyperkeratinization, thickening of the epidermis and loss of hair follicles with persistence of the sebaceous glands. Secondary bacterial infection occurred later.

The cornea of two animals showed vascularization and leucocytic infiltration similar to that which has been described in riboflavin deficiency. These ocular changes may indicate that zinc deficiency impairs the absorption or utilization of riboflavin.

The technical work was performed by Miss Miriam C. Reed.

#### LITERATURE CITED

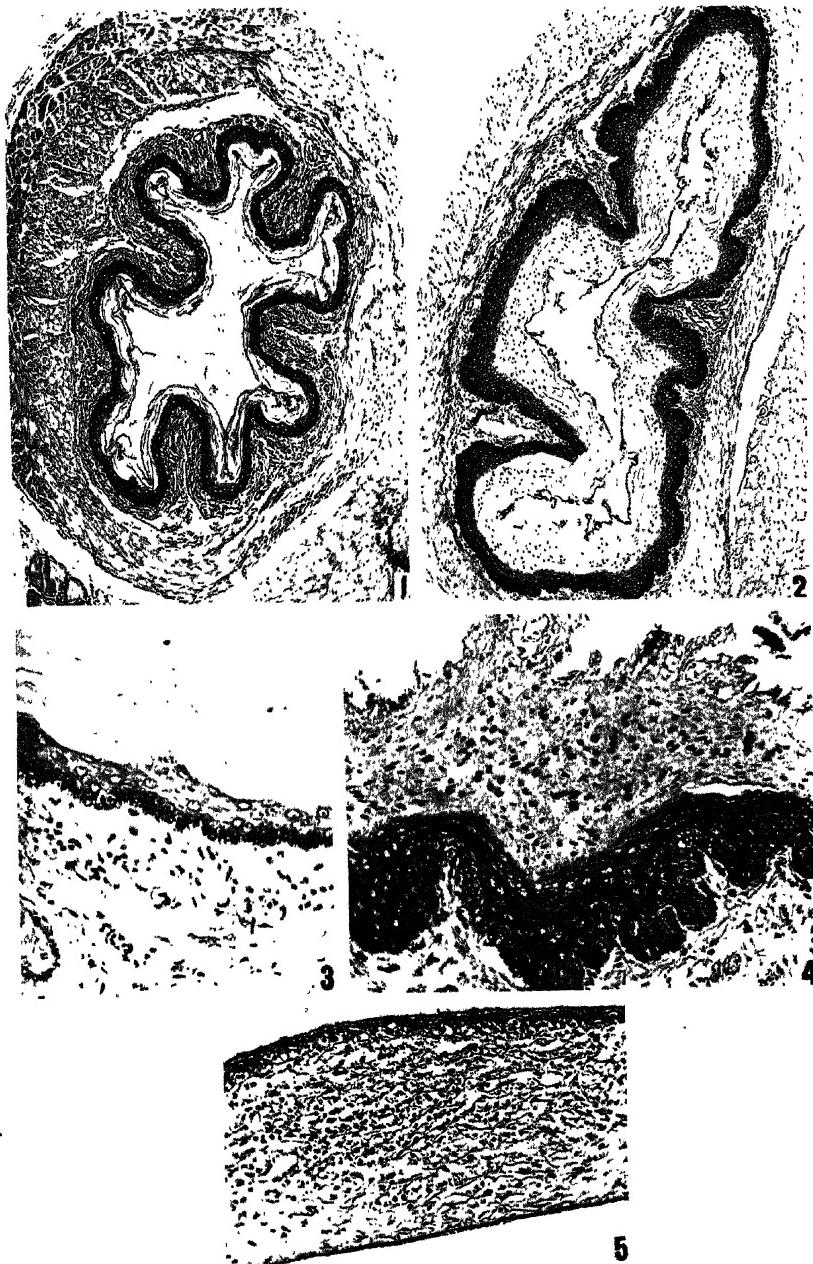
- BESSEY, O. A., AND S. B. WOLBACH 1939 Vascularization of the cornea of the rat in riboflavin deficiency with a note on corneal vascularization in vitamin A deficiency. *J. Exp. Med.*, vol. 69, p. 1.
- DAY, H. G., AND E. V. MCCOLLUM 1940 Effects of acute dietary zinc deficiency in the rat. *Proc. Soc. Exp. Biol. and Med.*, vol. 45, p. 282.
- HOVE, E., C. A. ELVEHJEM AND E. B. HART 1937 The physiology of zinc in the nutrition of the rat. *Am. J. Physiol.*, vol. 119, p. 768.
- 1938 Further studies on zinc deficiency in rats. *Am. J. Physiol.*, vol. 124, p. 750.
- 1940 a The effect of zinc on alkaline phosphatases. *J. Biol. Chem.*, vol. 134, p. 425.
- 1940 b The relation of zinc to carbonic anhydrase. *J. Biol. Chem.*, vol. 136, p. 425.
- GYORGY, P., M. SULLIVAN AND H. T. KARSNER 1937 Nutritional dermatoses in rats. *Proc. Soc. Exp. Biol. and Med.*, vol. 37, p. 313.
- STIRN, F. E., C. A. ELVEHJEM AND E. B. HART 1935 The indispensability of zinc in the nutrition of the rat. *J. Biol. Chem.*, vol. 109, p. 347.
- SULLIVAN, M., AND J. NICHOLLS 1940 a The nutritional approach to experimental dermatology. *J. Invest. Dermatol.*, vol. 3, p. 309.
- 1940 b The nutritional approach to experimental dermatology. Nutritional dermatoses in the rat. I. Vitamin B<sub>6</sub> deficiency. *J. Invest. Dermatol.*, vol. 3, p. 317.
- 1941 Nutritional dermatoses in the rat. Riboflavin deficiency. *J. Invest. Dermatol.* In press.
- THEORELL, H. 1940 Non-proteolytic enzymes. In *Annual Review of Biochemistry*, vol. 9, p. 670. Edited by J. M. Luck, Stanford University, P. O., California.
- TODD, W. R., C. A. ELVEHJEM AND E. B. HART 1934 Zinc in the nutrition of the rat. *Am. J. Physiol.*, vol. 107, p. 146.
- WOLBACH, S. B., AND P. R. HOWE 1925 Tissue changes following deprivation of fat soluble A vitamin. *J. Exp. Med.*, vol. 42, p. 753.

PLATE 1

EXPLANATION OF FIGURES

Photographs by Mr. Milton Kougl

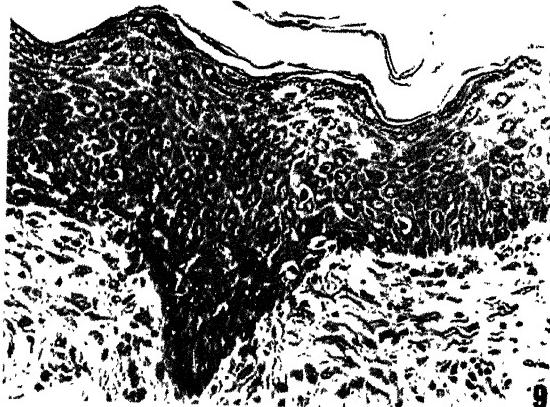
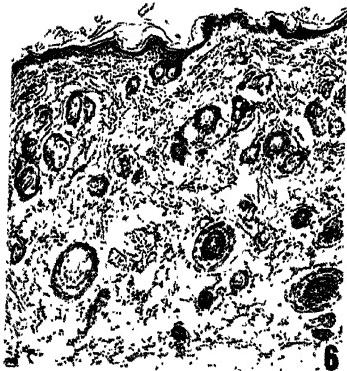
- 1 Low power photomicrograph of normal oesophagus.
- 2 Lower power photomicrograph of oesophagus from Zn-deficient rat. Same magnification as figure 1.
- 3 Higher power photomicrograph of normal lining of oesophagus.
- 4 Higher power photomicrograph of lining of oesophagus of Zn-deficient animal. Same magnification as figure 3.
- 5 Photomicrograph of cornea of one of the two animals showing vascularization with leucocytic infiltration.



## PLATE 2

### EXPLANATION OF FIGURES

- 6 Photomicrograph of skin of normal rat showing hair follicles and small sebaceous glands.
- 7 Photomicrograph of skin of Zn-deficient rat to show absence of hair follicles, enlargement of sebaceous glands, and thickening of epidermis.
- 8 Higher power photomicrograph of normal skin.
- 9 Higher power photomicrograph (same magnification as fig. 8) of Zn-deficient rat's epithelium.
- 10 High power photomicrograph of Zn-deficient rat's skin to show inter-cellular oedema and leucocytic infiltration.
- 11 Photomicrograph of skin of nose of Zn-deficient rat to show keratinization and crust formation.





## EDITORIAL REVIEW

### THE NUTRITIONAL IMPORTANCE OF CHOLINE<sup>1</sup>

WENDELL H. GRIFFITH

*Department of Biological Chemistry, St. Louis University School of Medicine,  
St. Louis*

(Received for publication February 10, 1941)

#### *Relation of choline and of protein to liver lipids in older rats*

The effects of choline deficiency which were recognized first and which, therefore, have been studied most extensively involve changes in the distribution of lipids in the animal body. As a primary result of the absence of dietary choline and of its precursors, neutral fat accumulates in the liver of the depancreatized dog and of the intact rat. The production of a "fatty liver" in the rat and its prevention by the inclusion of lecithin or of choline in the food mixture were reported by Best, Hershey and Huntsman ('32 a and b) following the demonstration, in depancreatized dogs, of the lipotropic action of raw pancreas (Allan et al., '24) and of lecithin (Hershey, '30; Hershey and Soskin, '31; Best and Hershey, '32). Best and Huntsman noted the choline-like action of betaine ('32) and the effectiveness of choline in

<sup>1</sup> This review has been limited purposely to the evidence of the dietary indispensability of choline, to its relation to other constituents of food mixtures and to the implications of its functions in metabolism. It has not been possible to include the extensive literature dealing with the activity of analogues of choline and of related chemical compounds (see Best and Ridout, '39) or with the significance of acetylcholine in myoneural physiology (see Alles, '34). Brief reference only has been made to the relation of choline to the transportation and utilization of lipids (see Best and Ridout, '39; Bloor, '40) and to its relation to the pancreatic factor, lipoenaeic (see Dragstedt, '40).

causing the disappearance of fat previously deposited in the livers of rats fed the low choline diet ('35). In these experiments fatty livers were produced in 3 weeks in 150 to 250 gm. rats on a mixed grain ration containing added fat.

The lipotropic action of casein was recognized by Best and Huntsman ('35) in experiments in which rats were first fed the above-mentioned high fat, low choline diet and then fed sucrose or sucrose and casein (20%). An additional deposition of liver fat occurred in the animals fed sucrose but not in those fed sucrose and casein. Channon and Wilkinson ('35) confirmed this effect of casein and introduced the low casein (5%), high fat (40%) diet which was widely adopted in the study of choline. The choline-like action of 1 gm. of casein was reported equivalent to 5 to 6 mg. of choline by Best, Grant and Ridout ('36) and to 7 to 8 mg. of choline by Beeston, Channon et al. ('36).

The addition of cholesterol to the diet of rats results in a "cholesterol fatty liver" in which there is a marked increase in cholesterol as well as in glycerol esters (Blatherwick et al., '33; Okey et al., '34). Best, Channon and Ridout ('34) demonstrated that supplements of choline in diets containing cholesterol completely prevent the deposition of glycerides but only partially prevent the accumulation of cholesteryl esters. Similar effects resulted from increases in the level of dietary casein (Beeston et al., '35; Best and Ridout, '36).

Synthesis of fat occurs in rats on low choline low fat rations (Longenecker et al., '40) and there is no evidence that the rate of oxidation of fat is seriously impaired by the lack of choline (Deuel et al., '37). The accumulation of glycerides in the liver probably results from a failure of transportation of fatty acids from this organ due to an inadequate supply of the choline required for the synthesis of the choline phospholipids. Although the phospholipids are generally believed to play an important part in the transportation of fatty acids (Sinclair, '34; Bloor, '40), the total amount of these phosphorylated compounds in liver remains relatively constant if calculated on a fat-free, dry weight

basis (MacLachlan and Hodge, '39). This constancy is also emphasized by Chaikoff who has demonstrated a marked stimulation of phospholipid "turnover" in the livers of rats following the administration of choline (Perlman and Chaikoff, '39 a and b). In view of the fact that a continuous supply of new choline appears to be essential for the movement of fatty acids out of the liver, it seems evident that a part of the choline used in such phospholipids is catabolized and does not return to the liver. No such direct explanation is acceptable, as yet, for the accumulation of cholesterol esters. Evidence which will be cited later points to the existence of other factors in addition to choline which are concerned with the metabolism of cholesterol.

*Relation of choline, cystine and methionine to liver lipids  
in older rats*

In order to determine which, if any, of the amino acids of casein might be responsible for the marked lipotropic activity of this protein, Beeston and Channon ('36) initiated the supplementation of the low casein ration with amino acids and found that cystine produces a surprising increase in the deposition of liver fat. In this connection, it is of interest that dietary cystine had previously been found to produce fatty degeneration of the liver in experiments which were not at the time related to choline metabolism (Curtis and Newburgh, '27; Lillie, '32). Tucker and Eckstein ('37) confirmed the results of Beeston and Channon and demonstrated that methionine exerts an opposite effect and is definitely lipotropic. Tucker et al. ('40) later found that if a 5% casein or edestin diet was supplemented with the amounts of methionine and cystine present in a 20% casein diet, the resulting lipotropic activity equalled that of the casein diet. This effect of methionine was confirmed by Channon, Manifold and Platt ('38) and by Best and Ridout ('40) although the latter questioned the conclusion that methionine was solely responsible for the lipotropic activity of casein. It is pertinent to note that in many of these experiments the dietary protein

was deficient in amount and in quality, a point recognized by Channon, Loach et al. ('38). It will be shown later that the symptoms of choline deficiency in young rats are affected by the nutritive state and that the cystine effect may only be a manifestation of an existing shortage of cystine or of total sulfur amino acids as well as of choline. Channon et al. ('40) have reported that amounts of cystine in excess of 4 mg. per day are no more effective than the 4 mg. level in causing an increased deposition of liver fat, an observation which suggests that the cystine effect may not be due to the specific interrelationship of cystine and choline.

#### *Relation of choline to methylation of homocysteine*

Additional evidence of the metabolic interrelationship of choline and of methionine is afforded by the investigations of du Vigneaud and his coworkers who demonstrated that supplements of choline or betaine in a diet which contained homocystine but which was devoid of methionine and cystine stimulated the growth of young rats (du Vigneaud, Chandler et al., '39). In the earlier experiments of White and Beach ('37) homocystine improved a low methionine diet and these workers suggested the conversion of homocystine into methionine. Later, du Vigneaud, Dyer and Kies ('39) reported that homocystine permitted growth on a diet otherwise devoid of sulfur amino acids if tikitiki and milk vitamin concentrate were used as sources of the vitamin B complex but not if a mixture of pure vitamins was supplied, and Rose and Rice ('39) reported similar results. Having noted that fatty livers occurred in his experimental animals, du Vigneaud added choline to the purified vitamin supplement and demonstrated, as cited above, that homocystine and choline may be substituted for methionine (and cystine). The study by du Vigneaud, Chandler et al. ('39) demonstrated the presence of choline in the material used as a source of the B complex in the experiments in which homocystine had been found to stimulate growth. In subsequent experiments du Vigneaud et al. ('40) showed by means of isotopic hydrogen that this element,

which was administered to rats in the methyl group of methionine, could be found later in tissue choline and creatine. As a result of these remarkable observations, du Vigneaud concluded that transmethylation, the specific transfer of methyl groups, occurs in the animal organism; that the methyls of methionine, choline and betaine are transferable; and, that methyl groups in such utilizable forms may be essential in the diet. Welch ('41) has reported the preparation of a methionine-free hydrolysate of casein as a substitute for the mixture of pure amino acids used by Rose and by du Vigneaud in the study of the metabolism of homocystine.

*Relation of choline to hemorrhagic degeneration in  
young rats*

The importance of dietary choline is further emphasized by the investigations of Griffith et al., who described an acute pathological state, hemorrhagic degeneration, in young male rats on diets which appeared adequate in protein and in dietary essentials other than choline. On these diets, rats, 21 to 26 days of age, developed fatty livers within 48 hours and severe hemorrhagic renal degeneration, ocular hemorrhage and regression of the thymus within 10 days (Griffith and Wade, '39 and '40; Griffith, '40 b). The results were spectacular not only because of the rapid onset of these effects but also because of the equally rapid "recovery" occurring in those rats which survived. The renal hemorrhage disappeared in the recovery phase but the fatty liver and signs of renal pathology persisted. With the exception of the new manifestations of the lack of choline, the metabolic aspects of this deficiency in young rats resembled those involved in the occurrence of fatty livers in older rats. Hemorrhagic degeneration was prevented by choline, methionine and betaine (Griffith, '41; Griffith and Mulford, '41 a) and was aggravated by cystine and cholesterol (Griffith, '40 a; Griffith and Mulford, '41 b). It was evident in these experiments that the choline requirement of young growing rats is greater than that of older animals since the severe effects appeared on

diets containing amounts of casein (15 to 25%) which are protective if fed to older animals.

The severe renal pathology occurring in young rats on rations deficient in choline (and low in methionine) had apparently been noted previously but had not been correlated with the lack of choline (Hartwell, '28; Cox et al., '29; Cox and Hudson, '30). Discoloration of the kidneys was observed by du Vigneaud, Dyer and Kies ('39) and du Vigneaud, Chandler et al. ('39) reported renal hemorrhage in one rat on the diet containing homocystine as the source of the sulfur amino acids. Inasmuch as the 18% casein diet does not supply sufficient methionine to prevent choline deficiency, it may appear surprising that the renal effects have not been noted more frequently in nutritional investigations. It is of interest in this connection that the deficiency is less severe in rats of both sexes if over 30 days of age and in female rats less than 30 days of age (Griffith, '40 b) and that severe renal hemorrhage has not been observed, even in young male rats, if the sucrose of the basal ration is replaced by starch (unpublished experiments). An additional finding, which is believed to be of considerable importance in the interpretation of results concerned with the requirement of choline, is the fact that the renal pathology and fatty liver do not occur if there is restriction of the intake of a food mixture which produces severe damage when fed ad libitum.

This direct relation between the rate of metabolism or of growth and the need of choline is also believed to be the basis for the apparent antagonism between this substance and cystine. In seeking an explanation for the aggravating effect of supplements of cystine in the 18% casein diet and particularly for the fact that 1% of added cystine was no more injurious than 0.3%, the suggestion was made that 18% casein is an inadequate source of sulfur amino acids, that added cystine prevents cystine deficiency, and that extra choline is then required because of the improved nutritional state (Griffith, '41). Definite evidence is now available (Mulford and Griffith, unpublished experiments) that the 18%

casein diet which is supplemented with choline is significantly improved by the addition of cystine. This improvement is characterized by greater increases in the body weight and length of the experimental rats and particularly by an increased efficiency of utilization of food.

*Relation of choline to thiamine and other vitamins of the B complex*

Supplements of thiamine in low choline diets increased the deposition of liver fat in rats, and McHenry ('37 a and b) concluded that this vitamin and choline are antagonistic and that both are required for the normal utilization of fats. Gavin and McHenry ('40) noted that supplementary pyridoxine ( $B_6$ ) and nicotinic acid slightly augment the body fat of rats on low-choline diets but that neither these two vitamins nor riboflavin prevent the deposition of liver fat which results from the administration of thiamine. The amount of liver fat was normal if choline was administered, either alone or with any combination of the three vitamins. Halliday ('38) reported that pyridoxine deficiency in rats aggravated the effect of choline deficiency with respect to deposition of liver fat and Gyorgy and Goldblatt ('40) found that renal hemorrhagic lesions occurred more frequently if the food mixture contained pyridoxine.

Griffith and Mulford ('41 b) were unable to demonstrate any effect of supplements of thiamine or of pyridoxine on liver fat in experiments in which these compounds were added to a diet which contained yeast and which was adequate for growth. The previously cited protective effect of subnormal metabolism induced by restriction of food intake raises the question as to whether the results of McHenry and of Gyorgy really represent direct antagonisms of thiamine and of pyridoxine to choline. Dietary supplements which improve deficient or even suboptimal diets may appear to act in direct opposition to choline. Such an effect may be nonspecific and have no direct relation to the metabolism of choline itself.

*Relation of choline to phospholipid turnover in the liver*

Chaikoff has used radioactive phosphorus ( $P^{32}$ ) to advantage in the study of the metabolism of the phospholipids. Choline accelerates the formation and removal of phospholipids in rat livers (Perlman and Chaikoff, '39 a). This effect is evident within 1 hour and is proportional to the amount of choline administered. The phospholipid turnover is also stimulated by betaine (Perlman and Chaikoff, '39 b) and by cystine and methionine (Perlman et al., '40 a and b). Cholesterol markedly decreases the turnover and the stimulating effect of choline is more evident in cholesterol-fed than in fat-fed rats (Perlman and Chaikoff, '39 c). Fries et al. ('40) studied the effect of age on phospholipid turnover in the central nervous system of rats and found that the activity is maximal on the first day of life and that it decreases to a low level by the time the rat weighs 50 gm. This is of interest in view of the fact that Griffith ('40 b) found a marked decrease in the choline requirement of rats over 30-35 days of age. Chanutin ('31) has concluded that young rats reach "chemical maturity" between 30 and 40 days of age.

*Relation of choline to manganese in the prevention of perosis in birds*

The role of manganese in the prevention of perosis (slipped tendon disease) was indicated by the observations of Wilgus et al. ('36) and Insko et al. ('38). Later, Hogan et al. ('40) reported that perosis, which occurred in spite of adequate dietary manganese, was prevented by some constituent of an alcoholic extract of liver. Jukes ('40 a and b, '41) found that choline prevented perosis and stimulated the growth of young turkeys and chicks on rations supplemented with manganese and that betaine was ineffective as a substitute for choline in such experiments. The antiperotic action of choline was confirmed by Hogan et al. ('41) and by Hegsted et al. ('41).

*Other effects of choline*

The administration of choline depresses hematopoiesis, induced by cobalt, in dogs (Davis, '39); prevents papillomatous lesions in the forestomach of rats on a diet containing 89% white flour (Sharpless, '40); is indispensable for lactation in adult rats and for growth and prevention of paralysis in suckling rats (Sure, '40); and, is essential for normal nutrition and egg production in chickens (Abbott and DeMasters, '40). Bloor ('40) has emphasized the relation of choline and of lecithin to acid-base equilibria and to the excretion of phosphate by the kidneys.

*Relation of choline to unidentified dietary factors concerned with the metabolism and transport of lipids*

The complexities of lipid metabolism are only partly clarified by the demonstrated effects of dietary choline. Evidence is increasing for the existence of one or more additional compounds which have a role in the choline-lipid interrelationship. Some of this evidence is as follows: on certain basal diets, supplements of methionine and cystine are not equal in lipotropic activity to equivalent amounts of these acids supplied as casein (Best and Ridout, '40); the accumulation of cholesterol esters is only partially prevented by supplements of choline in diets containing cholesterol (Blatherwick et al., '33); raw pancreas is more effective than choline in preventing the decrease in the lipids of the blood of dogs after ligation of the pancreatic ducts (Entenman et al., '39, '40 and '41); the accumulation of liver lipids induced by an aqueous extract of liver is not prevented by choline (Blatherwick et al., '33) but is prevented by lipocaic (McHenry and Gavin, '40); a pancreatic fraction, lipocaic may be more effective than choline in the prevention of fatty degeneration of the liver (Dragstedt, '40); and, renal and ocular hemorrhage occur in rats after 30 to 40 days on a diet containing cholesterol and choline even though protection is afforded during the crucial 6 to 10 day period (Griffith

and Mulford, '41 b). In the latter experiments, the similarity in the hemorrhagic lesions found at the end of the shorter period in the absence of choline and at the end of the longer period in the presence of choline suggests that these effects are not due to the lack of labile methyl alone. It may be that choline serves as one precursor of the lecithin required for the prevention of the fatty liver and, in addition, is used as choline, or as a source of methyl, in the synthesis of an unrecognized compound needed particularly in the metabolism of cholesterol. Such a compound, or its precursor, might be supplied by the diet and by the tissues of the rat in sufficient amounts during the 6 to 10 day period on a diet containing choline but not during a 30 to 40 day period. It is noteworthy that the later occurrence of renal hemorrhage on the cholesterol diet containing choline was not associated with marked deposition of liver lipids.

The choline-like action of methionine and betaine appears satisfactorily explained on the basis of a transfer of methyl groups by the process of transmethylation proposed by du Vigneaud. Other compounds having monomethyl and even trimethyl (betaine) groups attached to nitrogen are known to occur in animal tissues. The metabolic significance of these substances, ergothioneine and carnitine, for instance, remains to be determined.

#### *The daily requirement of choline in young rats*

Best and Huntsman ('35) proposed that choline be considered a dietary essential. Such a conclusion is justifiable even though signs of choline deficiency may be prevented by methionine or by betaine and even though synthesis of choline may occur in the rat (Jacobi et al., '41). The demonstrated value of dietary choline in rats and chicks is evidence of the inability of the organism to supply its own choline in adequate amounts by synthesis. Excessive levels of even high methionine proteins, such as casein, are necessary if sufficient methionine is to be supplied in the form of protein. Betaine is less effective than choline in the prevention of hemorrhagic

degeneration (Griffith and Mulford, '41 a) and as a supplement in a diet containing homocystine as the sole sulfur amino acid (Chandler and du Vigneaud, '40). Although it is logical to believe that choline is needed as such in view of its occurrence in the choline phospholipids, it is probable that it also serves in normal metabolism as a source of labile methyl in the synthesis of other important compounds.

The daily requirement of choline may be stated with respect to a specific food mixture only, since it varies with the dietary methionine, cystine, betaine and cholesterol as well as with the adequacy of the ration for optimum growth. The requirement has been determined by Griffith and Mulford (unpublished experiments) in male rats, 40 gm. in weight and 21 to 26 days of age, fed a basal diet with the following percentage composition: casein 6 to 42, lard 19, inorganic salts 5, agar 2, yeast 6, fortified fish liver oil 1 and sucrose 25 to 61. Very high casein levels of 30% or more are necessary to supply sufficient methionine to permit omission of a choline supplement. On casein levels of 18 to 24%, hemorrhagic degeneration is prevented by 1 to 2 mg. of choline chloride daily and the deposition of liver lipids by 4 to 6 mg.

#### LITERATURE CITED

- ABBOTT, O. D., AND C. U. DEMASTERS 1940 Choline in the diet of chickens. *J. Nutrition*, vol. 19, p. 47.
- ALLAN, F. N., D. J. BOWIE, J. J. R. MACLEOD AND W. L. ROBINSON 1924 Behaviour of depancreatized dogs kept alive with insulin. *Brit. J. Exp. Path.*, vol. 5, p. 75.
- ALLES, G. A. 1934 The physiological significance of choline derivatives. *Physiol. Rev.*, vol. 14, p. 276.
- BEESTON, A. W., AND H. J. CHANNON 1936 Cystine and the dietary production of fatty livers. *Biochem. J.*, vol. 30, p. 280.
- BEESTON, A. W., H. J. CHANNON, J. V. LOACH AND H. WILKINSON 1936 Further observations on the effect of dietary caseinogen in prevention of fatty livers. *Biochem. J.*, vol. 30, p. 1040.
- BEESTON, A. W., H. J. CHANNON AND H. WILKINSON 1935 The influence of the caseinogen content of diets on the nature of the cholesterol fatty liver. *Biochem. J.*, vol. 29, p. 2659.
- BEST, C. H., H. J. CHANNON AND J. H. RIDOUT 1934 Choline and the dietary production of fatty livers. *J. Physiol.*, vol. 81, p. 409.

- BEST, C. H., R. GRANT AND J. H. RIDOUT 1936 The lipotropic effect of dietary protein. *J. Physiol.*, vol. 86, p. 337.
- BEST, C. H., AND J. M. HERSEY 1932 Further observations on the effects of some components of crude lecithine on depancreatized animals. *J. Physiol.*, vol. 75, p. 49.
- BEST, C. H., J. M. HERSEY AND M. E. HUNTSMAN 1932 a The effect of lecithine on fat deposition in the liver of the normal rat. *J. Physiol.*, vol. 75, p. 56.
- 1932 b The control of the deposition of liver fat. *Amer. J. Physiol.*, vol. 101, p. 7.
- BEST, C. H., AND M. E. HUNTSMAN 1932 The effects of the components of lecithine upon deposition of fat in the liver. *J. Physiol.*, vol. 75, p. 405.
- 1935 The effect of choline on the liver fat of rats in various states of nutrition. *J. Physiol.*, vol. 83, p. 255.
- BEST, C. H., AND J. H. RIDOUT 1936 Dietary casein and cholestryl esters in liver. *J. Physiol.*, vol. 87, p. 55P.
- 1939 Choline as a dietary factor. *Ann. Rev. of Biochem.*, vol. 8, p. 349.
- 1940 The lipotropic action of methionine. *J. Physiol.*, vol. 97, p. 489.
- BLATHERWICK, N. R., E. M. MEDLAR, P. J. BRADSHAW, A. L. POST AND S. D. SAWYER 1933 The dietary production of fatty livers in rats. *J. Biol. Chem.*, vol. 103, p. 93.
- BLOOR, W. R. 1940 Fat transport in the animal body. *Physiol. Rev.*, vol. 19, p. 557.
- CHANDLER, J. P., AND V. DU VIGNEAUD 1940 The comparative action of choline and betaine in effecting the replacement of methionine by homocystine in the diet. *J. Biol. Chem.*, vol. 135, p. 223.
- CHANNON, H. J., J. V. LOACH, P. A. LOIZIDES, M. C. MANIFOLD AND G. SOLIMAN 1938 Dietary proteins in fatty liver production. *Biochem. J.*, vol. 32, p. 976.
- CHANNON, H. J., M. C. MANIFOLD AND A. P. PLATT 1938 The action of cystine and methionine on liver fat deposition. *Biochem. J.*, vol. 32, p. 969.
- 1940 The action of sulphur-containing amino acids and proteins on liver fat deposition. *Biochem. J.*, vol. 34, p. 866.
- CHANNON, H. J., AND H. WILKINSON 1935 Protein and the dietary production of fatty livers. *Biochem. J.*, vol. 29, p. 350.
- CHIANTIN, A. 1931 The influence of growth on a number of constituents of the white rat. *J. Biol. Chem.*, vol. 93, p. 31.
- COX, G. J., AND L. HUDSON 1930 The nephropathogenic action of cystine. II. The dietary control of cystine nephrosis. *J. Nutrition*, vol. 2, p. 271.
- COX, G. J., C. V. SMYTHE AND C. F. FISHBACK 1929 The nephropathogenic action of cystine. *J. Biol. Chem.*, vol. 82, p. 95.
- CURTIS, A. C., AND L. H. NEWBURGH 1927 The toxic action of cystine on the liver of the albino rat. *Arch. Int. Med.*, vol. 39, p. 828.
- DAVIS, J. E. 1939 Depression of experimental polycythemias by choline hydrochloride or liver administration. *Amer. J. Physiol.*, vol. 127, p. 322.

- DEUEL, H. J., JR., S. MURRAY, L. F. HALLMAN AND D. B. TYLER 1937 Studies on ketosis. XII. The effect of choline on the ketonuria of fasting rats following a high fat diet. *J. Biol. Chem.*, vol. 120, p. 277.
- DRAGSTEDT, L. R. 1940 The present status of lipocaine. *J. Amer. Med. Assn.*, vol. 114, p. 29.
- DU VIGNEAUD, V., J. P. CHANDLER, M. COHN AND G. B. BROWN 1940 The transfer of the methyl group from methionine to choline and creatine. *J. Biol. Chem.*, vol. 134, p. 787.
- DU VIGNEAUD, V., J. P. CHANDLER, A. W. MOYER AND D. M. KEPPEL 1939 The effect of choline on the ability of homocystine to replace methionine in the diet. *J. Biol. Chem.*, vol. 131, p. 57.
- DU VIGNEAUD, V., H. M. DYER AND M. W. KIES 1939 A relationship between the nature of the vitamin B complex supplement and the ability of homocystine to replace methionine in the diet. *J. Biol. Chem.*, vol. 130, p. 325.
- ENTENMAN, C., I. L. CHAIKOFF AND M. L. MONTGOMERY 1939 The blood lipids of dogs subjected to ligation of the external pancreatic ducts. *J. Biol. Chem.*, vol. 130, p. 121.
- ENTENMAN, C., M. L. MONTGOMERY AND I. L. CHAIKOFF 1940 The effect of choline on the blood and liver lipids of the dog subjected to ligation of the pancreatic ducts. *J. Biol. Chem.*, vol. 135, p. 329.
- ENTENMAN, C., AND I. L. CHAIKOFF 1941 Is choline the factor in the pancreas that prevents fatty livers in depancreatized dogs maintained with insulin? *J. Biol. Chem.*, vol. 138, p. 477.
- FRIES, B. A., G. W. CHANGUS AND I. L. CHAIKOFF 1940 Radioactive phosphorus as an indicator of phospholipid metabolism. IX. Influence of age on the phospholipid metabolism of various parts of the central nervous system of the rat. The comparative phospholipid activity of various parts of the central nervous system of the rat. *J. Biol. Chem.*, vol. 132, p. 23.
- GAVIN, G., AND E. W. McHENRY 1940 The B vitamins and fat metabolism. III. The effects of vitamin B<sub>6</sub> upon liver and body fat. *J. Biol. Chem.*, vol. 132, p. 41.
- GRIFFITH, W. H., AND N. J. WADE 1939 Choline metabolism. I. The occurrence and prevention of hemorrhagic degeneration in young rats on a low choline diet. *J. Biol. Chem.*, vol. 131, p. 567.
- 1940 Choline metabolism. II. The interrelationship of choline, cystine and methionine in the occurrence and prevention of hemorrhagic degeneration in young rats. *J. Biol. Chem.*, vol. 132, p. 627.
- GRIFFITH, W. H. 1940 a Choline metabolism. III. The effect of cystine, fat and cholesterol on hemorrhagic degeneration in young rats. *J. Biol. Chem.*, vol. 132, p. 639.
- 1940 b Choline metabolism. IV. The relation of the age, weight and sex of young rats to the occurrence of hemorrhagic degeneration on a low choline diet. *J. Nutrition*, vol. 19, p. 437.
- 1941 Choline metabolism. V. The effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in young rats. *J. Nutrition*, vol. 21, p. 291.

- GRIFFITH, W. H., AND D. J. MULFORD 1941 a Choline metabolism. VI. Hemorrhagic degeneration and the labile methyl supply. *J. Amer. Chem. Soc.*, vol. 63, p. 929.
- 1941 b Choline metabolism. VII. Some dietary factors affecting the incidence and severity of hemorrhagic degeneration in young rats. *J. Nutrition*, vol. 21, p. 633.
- GYORGY, P., AND H. GOLDBLATT 1940 Choline as a member of the vitamin B<sub>1</sub> complex. *J. Exp. Med.*, vol. 72, p. 1.
- HALLIDAY, N. 1938 Fatty livers in vitamin B<sub>6</sub> deficient rats. *J. Nutrition*, vol. 16, p. 285.
- HARTWELL, G. A. 1928 Protein and vitamin B. *Biochem. J.*, vol. 22, p. 1212.
- HEGSTED, D. M., R. C. MILLS, C. A. ELVERHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. *J. Biol. Chem.*, vol. 138, p. 459.
- HERSHEY, J. M. 1930 Substitution of lecithin for raw pancreas in the diet of the depancreatized dog. *Amer. J. Physiol.*, vol. 93, p. 657.
- HERSHEY, J. M., AND S. SOSKIN 1931 Substitution of "lecithin" for raw pancreas in the diet of the depancreatized dog. *Amer. J. Physiol.*, vol. 98, p. 74.
- HOGAN, A. G., L. R. RICHARDSON AND H. PATRICK 1940 Relation of perosis to unrecognized vitamins. *J. Nutrition*, vol. 19, p. XIV.
- HOGAN, A. G., L. R. RICHARDSON, H. PATRICK AND H. L. KEMPSTER 1941 Perosis due to a vitamin deficiency. *J. Nutrition*, vol. 21, p. 327.
- INSKO, W. M., M. LYONS AND J. H. MARTIN 1938 The quantitative requirement of the growing chick for manganese. *J. Nutrition*, vol. 15, p. 621.
- JACOBI, H. P., C. A. BAUMAN AND W. J. MEEK 1941 The choline content of rats on various choline-free diets. *J. Biol. Chem.*, vol. 138, p. 571.
- JUKES, T. H. 1940 a Prevention of perosis by choline. *J. Biol. Chem.*, vol. 134, p. 789.
- 1940 b Effect of choline and other supplements in perosis. *J. Nutrition*, vol. 20, p. 445.
- 1941 Effects of choline, gelatin and creatine on perosis in chicks. *Pro. Soc. Exper. Biol. and Med.*, vol. 46, p. 155.
- LILLIE, R. D. 1932 Histopathologic changes produced in rats by the addition to the diet of various amino acids. *U. S. P. H. R.*, vol. 47, p. 83.
- LONGENECKER, H. E., G. GAVIN AND E. W. McHENRY 1940 Fatty acids synthesized by the action of thiamine. *J. Biol. Chem.*, vol. 134, p. 693.
- MACLACHLAN, P. L., AND H. C. HODGE 1939 The influence of cocaine feeding on the liver lipids of the white mouse. *J. Biol. Chem.*, vol. 127, p. 721.
- MCHENRY, E. W. 1937 a Vitamin B<sub>1</sub> and fatty livers. *J. Physiol.*, vol. 89, p. 287.
- 1937 b An effect of choline on the vitamin B<sub>1</sub>—sparing action of fats. *Biochem. J.*, vol. 31, p. 1616.
- MCHENRY, E. W., AND G. GAVIN 1940 The effects of liver and pancreas extracts upon fat synthesis and metabolism. *J. Biol. Chem.*, vol. 134, p. 683.
- OKEY, R., H. L. GILLUM AND E. YOKELA 1934 Factors affecting cholesterol deposition in the tissues of rats. I. Differences in the liver lipids of males and females. *J. Biol. Chem.*, vol. 107, p. 207.

- PERLMAN, I., AND I. L. CHAIKOFF 1939 a Radioactive phosphorus as an indicator of phospholipid turnover. V. On the mechanism of the action of choline upon the liver of the fat-fed rat. *J. Biol. Chem.*, vol. 127, p. 211.
- 1939 b Radioactive phosphorus as an indicator of phospholipid turnover. VII. The influence of cholesterol upon phospholipid turnover in the liver. *J. Biol. Chem.*, vol. 128, p. 735.
- 1939 c Radioactive phosphorus as an indicator of phospholipid turnover. VIII. The influence of betaine on the phospholipid activity of the liver. *J. Biol. Chem.*, vol. 130, p. 593.
- PERLMAN, I., N. STILLMAN AND I. L. CHAIKOFF 1940 a Radioactive phosphorus as an indicator of phospholipid metabolism. XI. The influence of methionine, cystine and cysteine upon the phospholipid turnover in the rat. *J. Biol. Chem.*, vol. 133, p. 651.
- 1940 b Radioactive phosphorus as an indicator of phospholipid metabolism. XII. Further observations on the effects of amino acids on phospholipid activity of the liver. *J. Biol. Chem.*, vol. 135, p. 359.
- ROSE, W. C., AND E. E. RICE 1939 Utilization of certain sulfur-containing compounds for growth purposes. *J. Biol. Chem.*, vol. 130, p. 305.
- SHARPLESS, G. R. 1940 Choline and epithelial hyperplasia in the forestomach of rats. *Proc. Soc. Exp. Biol. and Med.*, vol. 45, p. 487.
- SINCLAIR, R. G. 1934 The physiology of the phospholipids. *Physiol. Rev.*, vol. 14, p. 351.
- SURE, B. 1940 The essential nature of choline for lactation and growth of the albino rat. *J. Nutrition*, vol. 19, p. 71.
- TUCKER, H. F., AND H. C. ECKSTEIN 1937 The effect of supplementary methionine and cystine on the production of fatty livers by diet. *J. Biol. Chem.*, vol. 121, p. 479.
- TUCKER, H. F., C. R. TREADWELL AND H. C. ECKSTEIN 1940 The effect of supplementary cystine and methionine on the production of fatty livers by rats on high fat diets containing casein or edestin. *J. Biol. Chem.*, vol. 135, p. 85.
- WELCH, A. D. 1941 The preparation of a casein hydrolysate for the study of the relationship between choline and homocystine. *J. Biol. Chem.*, vol. 137, p. 173.
- WHITE, A., AND E. F. BEACH 1937 The role of cystine, methionine and homocystine in the nutrition of the rat. *J. Biol. Chem.*, vol. 122, p. 219.
- WILGUS, H. S., J. C. NORRIS AND G. F. HEUSER 1936 The role of certain inorganic elements in the cause and prevention of perosis. *Science*, vol. 84, p. 252.



## REDUCTION IN EXPERIMENTAL RAT CARIES BY FLUORINE<sup>1</sup>

SIDNEY B. FINN AND HAROLD C. HODGE

*Department of Biochemistry and Pharmacology, School of Medicine and Dentistry,  
The University of Rochester, New York*

SIX FIGURES

(Received for publication March 7, 1941)

Two recent independent observations on commercial casein as a dietary constituent are of interest: (a) that the commercial casein on the market sometime ago produced dental fluorosis in rats and was shown to contain about 0.2% of fluorine (Hodge, Luce-Clausen and Brown, '38), and (b) that commercial casein provided a protective action against experimental caries in rats (Lilly, '38). Combining these observations, the hypothesis was set up that fluorine was the factor supplying the protection. This hypothesis was the more tenable since both chemical analyses (Armstrong and Brekhus, '38), and clinical surveys (Dean et al., '39), had pointed to a relationship between caries activity and fluorine. Consequently groups of rats were fed diets containing "fluorine-free" casein with and without the addition of fluorides. Since this experiment was begun two reports have appeared (Miller, '38; Cox et al., '39), which demonstrated that fluorine inhibits experimental caries in rats. The data presented herewith not only confirm these findings but amplify those of Hodge and Finn ('39), and extend the previous observations.

<sup>1</sup>This work was supported in part by a grant from the Carnegie Corporation of New York. The data in this paper were taken from the thesis submitted by the senior author to the Committee on Graduate Studies of the University of Rochester in partial fulfillment of the requirements for the degree Master of Science.

## EXPERIMENTAL

One hundred and thirty-five rats (Wistar strain) were divided into three groups at weaning. The first group received the Hoppert, Webber and Canniff ('31) caries-producing diet,<sup>2</sup> the second group received the same diet with the substitution of commercial casein for the powdered whole milk (Lilly, '38), the third group received the latter diet with the addition of 3 mg. of fluorine (as KF) daily. The fluoride was given by pipette in a drop of water deposited on the tongue. The amount of fluorine is the same as would have been received had the dietary casein contained 0.2% fluorine. Each group of rats was subdivided into three groups; these received respectively, (a) corn particles (cracked corn) larger than 20 mesh only, (b) corn particles between 10 and 20 mesh, and (c) unsifted or run-of-the-mill cracked corn. The diets and distilled water were given ad libitum.

The rats were weighed every 4 days. The teeth were examined periodically using H. R. Hunt's technic (personal communication) for the evidence of carious destruction. Radiographs of the tibias on the fiftieth day using the technic of O'Brien and Morgareidge ('38) showed rickets in the rats receiving casein. This disease was promptly healed<sup>3</sup> (as shown by radiographs on the sixtieth day) through the incorporation of 2% whole yeast and 2% cod liver oil into the diets.

After 200 days on the diets, the rats were sacrificed, the jaws separated and studied in three ways. (1) Using a binocular microscope (15 X) the jaws were examined occlusally for caries and for fractures. (2) Through the kindness of Dr. G. J. Cox, the method of Cox and Dixon ('39) was applied to all the right jaws. This procedure detects and grades fissure caries by repeated successive grinding, staining and examination with reflected light (15 X). (3) The left jaws were prepared as ground and decalcified stained sections to observe

<sup>2</sup> Coarse corn meal 60%, powdered whole milk 30%, linseed meal 6%, alfalfa meal 3%, NaCl 1%.

<sup>3</sup> For radiographic details of the effect of fluorine on rickets, see Morgareidge and Finn ('40).

the nature and progress of the lesions. The results of these histological studies are to be presented elsewhere. In brief, these examinations gave indications as to the nature of rat caries but shed little light on the effect of fluorine.

### RESULTS

In figure 1 are given the data for the total incidence of caries in the molar teeth of the various groups of rats. The data are presented separately for each tooth type, e.g., first molar on the right side of the maxilla, et cetera. It is evident (1) that first molars (both upper and lower) have a higher incidence of

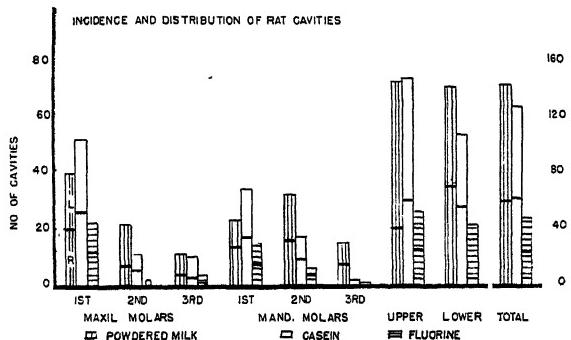


Fig. 1 The number of cavities is given as the ordinate; the data for each tooth type are given separately. The totals for the left side of the jaws are represented by the height above the horizontal dividing lines in each column, those for the right side by the lower part of each column. For each tooth, for the lower and upper jaws, and for the over-all totals, the fluorine-fed group had a lower caries incidence.

caries than the other molars although no molar type was completely caries-free; and (2) that for each tooth the total number of cavities was similar for the whole powdered milk and the casein fed groups but that a significant decrease in cavities was found in the group receiving fluorides. This fact is also demonstrated in the totals for upper and lower jaws and the over-all totals given on the right side of figure 1. The reduction in the number of cavities by fluorine (table 1) is shown to have a high probability of significance (table 2). The teeth of the upper jaw had more cavities (171) than those of the

TABLE 1  
Caries incidence for the various dietary groups and sub-groups.

DIETARY SUPPLEMENT	CORN PARTICLE SIZE	MESH	NO. RATS	NO. OF CAVITIES		NO. OF CUSPS INVOLVED		NO. OF TEETH DESTROYED		NO. CARIES-FREE MOUTHS
				total	per rat	total	per rat	total	per rat	
Powdered whole milk (30%)	Coarse	20	14	48	3.4	171	12	14	1.0	0
	Fine	10-20	13	55	4.2	143	11	10	0.8	0
	Run of mill	Unsifted	13	39	3.0	111	8	10	0.8	0
Totals and averages			40	142	3.5	425	11	34	0.9	0
Casein (30%)	Coarse	20	12	39	3.3	105	9	5	0.4	0
	Fine	10-20	15	45	3.1	147	10	10	0.7	0
	Run of mill	Unsifted	15	42	2.8	117	8	6	0.4	0
Totals and averages			42	126	3.0	369	9	21	0.5	0
Casein (30%) and fluorine (3 mg.)	Coarse	20	13	12	0.9	40	3	1	0.1	5
	Fine	10-20	14	19	1.3	50	4	1	0.1	3
	Run of mill	Unsifted	15	16	1.0	42	3	3	0.2	5
Totals and averages			42	47	1.1	132	3	5	0.1	13

TABLE 2  
Statistical analysis of data of table 1.

	POWDERED MILK	CASEIN	FLUORINE
No. cavities	142	126	47
No. cavities per rat	3.6	3.0	1.1
Compared to F group			
s <sup>1</sup>	1.23	1.06	
s <sub>x</sub>	0.27	0.23	
t	9.2	8.2	
N	80	82	
P	0.01	0.01	
No. cusps involved	425	369	132
% total cusps	19.0	15.7	5.6
Significance ratio <sup>2</sup> of mean differences with F group	14.1	11.4	
No. teeth destroyed	34	21	5
% total teeth	7.1	4.4	1.1
Significance ratio of mean differences with F group	5.0	3.1	
No. caries free mouths	0	0	13
% total mouths	0	0	31
Significance ratio of mean differences with F group	4.3	4.3	

<sup>1</sup> The statistical procedure used is given in Fisher ('34).

<sup>2</sup> The statistical procedure used is given in Yule and Kendall ('37). The "significance ratio" is equal to the actual difference in average percentages divided by the standard error of sampling.

lower jaw (145); calculations indicated that this difference would occur by chance twice out of ten trials. There was no regular tendency for an asymmetrical distribution of caries (right vs. left sides of the jaw).

The number of carious cusps for the various teeth is given in figure 2. The maxillary first molar is most frequently involved and, as before, there is evidence that the teeth of the upper jaws are more often carious. The reduction in the number of carious cusps in the rats receiving fluorine is clearly shown in the column for total values at the right. The probability that such a difference would occur by chance is very low (table 2).

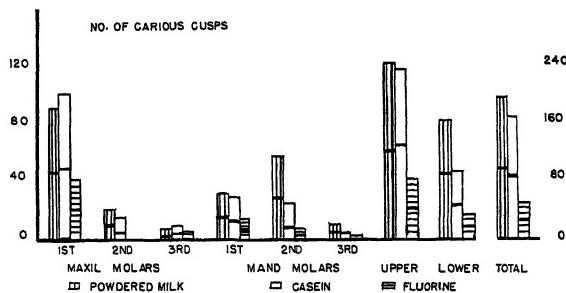


Fig. 2 The plotting is done as in figure 1. The reduction in the number of carious cusps of the fluorine-fed group is evident for each tooth type, for upper and lower jaws and for the over-all total.

Apparently, not only is the maxillary first molar the most frequent site of carious attack but the number of these teeth completely destroyed nearly equals the total for all the other molar types (fig. 3). What is meant by "completely destroyed" may be seen in figure 4; the crown of a mandibular second molar has been involved and has disappeared allowing the third molar to drift forward. The traumatic changes following the massive destruction of a tooth inevitably spread to the jaw bone as can be seen in the radiograph, figure 5. The inhibiting effect of fluorine is shown in the total number of teeth destroyed for the various groups, viz., milk—34, casein—21, fluorine—5.

The reduction by fluorine has a high statistical significance (table 2). Varying the particle size of the cracked corn had no significant effect on the caries incidence (table 1) whether the data are considered for number of cavities, number of teeth destroyed or number of cusps involved. It should be noted that all of the diets contained corn particles at least as large as 10 to 20 mesh, i.e., "coarse" corn particles.

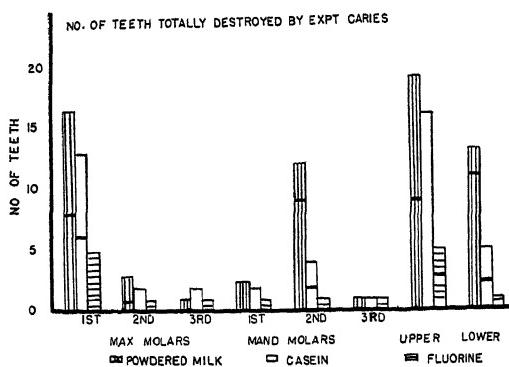


Fig. 3 The plotting is done as in figure 1. Note the very high destruction of maxillary first molars as well as the reduction of teeth destroyed in each case when fluorine was added to the diet.



Fig. 4 "Complete destruction" of a mandibular second molar. The third molar has drifted forward. Note the sharp cusps of the first molar and the evidence of fracture of the cusp nearest the second molar. (10 X, photograph by M. C. Orser.)

The growth curves for the male and female rats (fig. 6) show that the casein and fluorine groups lagged a little behind the milk-fed group in body weight; this may in part be due to the deficiency already described which was corrected on the



Fig. 5 Rat skulls are separated longitudinally into halves by the technic of B. J. Frey (personal communication) and radiographed. The upper skull shows the complete destruction of the first molar and the concomitant involvement of the adjacent alveolar bone. The lower skull is from a normal rat. (Print by P. S. S. Sweet, Eastman Kodak Co.)

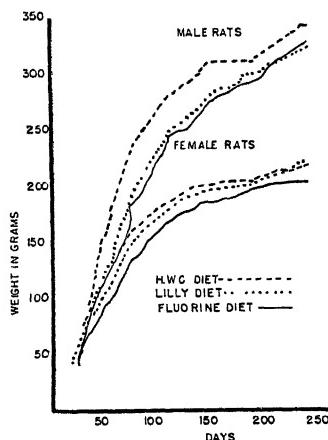


Fig. 6 Composite growth curves of the male and female rats on the diets indicated. The fluorine-fed rats grew well and gave no indication of a pronounced toxic effect.

fiftieth day. In general, the rats grew well, the fluorine-fed group giving no indication of a toxic effect on growth. The earliest indication of macroscopic caries was observed between the forty-fourth and fiftieth day on the diet. The seventy-two male rats showed the same caries incidence as the fifty-two female rats; these comparisons were made group by group.

The results of the examination of the teeth for fissure caries (through the cooperation of G. J. Cox) were surprising. In only seven teeth, four of which were lower first molars, were suspicious areas found. Of these, four were probably artifacts and only three could be classed as true fissure caries. Perhaps the long duration of the experiment permitted other fissure caries to coalesce with or simulate occlusal cavities and thus prevent identification.

Summarizing the effects (table 1) attributed to the fluorine in the diet: (1) there were on the average 1.1 cavities per rat fed fluorine; 3.5 per rat not fed fluorine. (2) Rats fed fluorine averaged 3 cusps involved per rat; the other two groups averaged 11 and 9 per rat. (3) On the average the fluorine-fed rats lost 0.1 tooth per rat; the other groups lost 0.9 and 0.5 tooth per rat. It is noteworthy that thirteen of the forty-two rats receiving fluorine had caries-free mouths; by sharp contrast, none of the eighty-two rats in the other groups had a caries-free mouth. This difference is statistically significant (table 2).

#### DISCUSSION

It may almost be said that every element known to exist in dental tissues has at one time been suspected as the cause of dental caries. However, various authors have differed in ascribing the disease on the one hand to an excess and on the other to a deficiency of the element in question. Fluorine is no exception; Sir Crichton-Browne (1892) suggested that a fluorine deficit might be the causal agent since, on the basis of earlier analyses, teeth were shown to be comparatively rich in fluorine. During the next decade Wrampelmeyer (1893) and Hempel and Scheffler (1899) reported higher fluorine values in sound than in carious teeth; in the light of present knowl-

edge the high method error invalidates their data. Only recently was a fluorine method of proved accuracy developed and a significantly higher fluorine content established for the enamel of sound teeth by Armstrong ('37) and Armstrong and Brekhus ('38). From their data the need of precise information is emphasized by two points. First, minute quantities of fluorine are present in tooth tissues. A 2-gm. tooth has perhaps 700 mg. of enamel; of this only 0.01% is fluorine or 70  $\mu\text{g}$ . Second, the enamel of a sound 2-gm. tooth would contain on the average about 30  $\mu\text{g}$ . more fluorine than would be found in the enamel of a carious tooth of the same weight.

There is evidence from the data of table 1 that fluorine limits caries progress as well as reduces the incidence of lesions. Fluorine-fed rats developed almost half as many cavities as the casein-fed controls (1:2.7), but the size of the cavities was less. Thus, for each cusp involved in the fluorine group, there were 3.6 cusps involved in the control group. The limitation of caries extension is further shown by the relative numbers of teeth destroyed; for each tooth destroyed in the fluorine-fed group, 4.2 teeth of the controls were destroyed. In the occlusal examination, fractured cusps which showed no concomitant staining or other signs of carious attack were not counted as cavities. There was little, if any, qualitative difference in the appearance of fractures in the teeth of the fluorine-fed group as compared to the controls; all the rats exhibited many fractured cusps. The data given above lead to the hypothesis (1) that fluorine prevents the development of a carious lesion on the site of a fractured cusp, thus giving the caries-free mouths; and (2) that fluorine prevents the development of a small cavity into a larger one, thus giving the lesser number of cusps involved and whole teeth destroyed.

While the experiment reported in this paper was being carried out, Miller ('38) reported that fluorides or iodoacetate added to the diet of rats produced a marked reduction in experimental caries. The data he presented from the lower molar teeth of fifty rats fed for 100 days on caries producing

diet showed that calcium fluoride gave an 81% reduction, sodium fluoride 94% and iodoacetic acid 100% reduction in caries incidence. That only 70% reduction is shown in table 1 may in part be attributed to the longer duration of this experiment (200 days) and in part to the inclusion of both upper and lower molars in the totals. However, in general, these results confirm the work of Miller ('38). Cox and co-workers ('39) feeding much less fluorine (20 p.p.m. of the diet) than Miller and ourselves did (ca. 300 p.p.m.) showed statistical evidence of a reduction in caries less dramatic than the reduction shown in table 1 or by Miller ('38).

It is difficult to make even tentative statements about the site of fluorine action or the mechanism of the inhibition of experimental caries. Rat caries as a phenomenon is not perfectly understood; there has been a long controversy over the manner of initiation and progress of the lesions, especially as to whether the enamel is the initial site of attack. If enamel caries is the first step then surface phenomena, such as the absorption of fluorides as shown by Volker et al. ('40), and repression of bacterial acid production as shown by Bibby and Van Kesteren ('40) would assume prime importance. If the initial lesion is a cusp fracture thereby exposing dentine and the subsequent course is a true dentinal caries, then in addition to the above-mentioned factors, the systemic and metabolic effects of fluorine and the defense mechanisms of the dentine would be involved. Volker ('39) has demonstrated not only that the lactic acid solubility of powdered normal enamel and dentine is reduced by a preliminary treatment with a solution of fluorides, but also that the enamel and dentine from rats fed fluorides were less soluble than these tissues from normal rats. The idea that solubility reduction and bacterial enzyme inhibition are major factors in the reduction of experimental rat caries must at present be regarded as conjecture; the mechanism of the inhibiting or protective action of fluorine is unknown.

## LITERATURE CITED

- ARMSTRONG, W. D. 1937 Fluorine content of enamel and dentine of sound and carious teeth. *J. Biol. Chem.*, vol. 119, p. v.
- ARMSTRONG, WALLACE D., AND P. J. BREKHSUS 1938 Possible relationship between the fluorine content of enamel and resistance to dental caries. *J. Dent. Res.*, vol. 17, p. 393.
- BIBBY, B. G., AND MARY VAN KESTEREN 1940 The effect of fluorine on mouth bacteria. *J. Dent. Res.*, vol. 19, p. 391.
- COX, G. J., M. C. MATUSCHAK, S. F. DIXON, M. L. DODDS AND W. E. WALKER 1939 Fluorine and its relation to dental caries. *J. Dent. Res.*, vol. 18, p. 481.
- COX, G. J., AND S. F. DIXON 1939 A method of grinding rat molars for observing fissure caries. *J. Dent. Res.*, vol. 18, p. 153.
- CRICHTON-BROWNE, J. 1892 An address on tooth culture. *Lancet*, vol. 2, p. 6.
- DEAN, H. T., PHILIP JAY, FRANCIS A. ARNOLD, JR., FRANK J. MCCLURE AND ELIAS ELVOVE 1939 Domestic water and dental caries including certain epidemiological aspects of oral *L. acidophilus*. *U. S. Pub. Health Reports*, vol. 54, p. 862.
- FISHER, R. A. 1934 Statistical Methods for Research Workers. Oliver and Boyd, London, 5th ed., p. 120.
- HEMPEL, W., AND W. SCHIEFFLER 1899 Über eine methode für Bestimmung des Fluors neben Kohlensäure und den Fluorgehalt von einigen Zähnen. *Zeitschrift fur anorganische Chemie*, vol. 20, p. 1.
- HODGE, HAROLD C., E. M. LUCE-CLAUSEN AND E. F. BROWN 1938 Fluorosis in rats due to contamination with fluorine of commercial casein. The effects of darkness and of controlled radiation upon the pathology of the teeth. *J. Nutrition*, vol. 17, p. 333.
- HODGE, HAROLD C., AND SIDNEY B. FINN 1939 Reduction in experimental rat caries by fluorine. *Proc. Soc. Exp. Biol. and Med.*, vol. 42, p. 318.
- HOPPERT, C. A., P. A. WEBBER AND T. L. CANNIFF 1931 The production of caries in rats fed an adequate diet. *Science*, vol. 74, p. 77.
- LILLY, C. A. 1938 Lessened incidence of caries replaces milk in the coarse corn meal diet. *Proc. Soc. Exp. Biol. and Med.*, vol. 38, p. 398.
- MILLER, BENJAMIN F. 1938 Inhibition of experimental dental caries in the rat by fluoride and iodoacetic acid. *Proc. Soc. Exp. Biol. and Med.*, vol. 39, p. 389.
- MORGAREIDGE, KENNETH, AND SIDNEY B. FINN 1940 Effect of fluorine on the activity of vitamin D in rachitic rats. *J. Nutrition*, vol. 20, p. 75.
- O'BRIEN, B., AND KENNETH MORGAREIDGE 1938 Radiographic demonstration of protection by vitamin D against metaphyseal decalcification in adult rats on high calcium-low phosphorus diet. *J. Nutrition*, vol. 16, p. 91.

- VOLKER, J. F., H. WILSON, HAROLD C. HODGE AND S. N. VAN VOORHIS 1940 The adsorption of fluoride by enamel, dentin, bone and hydroxyapatite as shown by the radioactive isotope. *J. Biol. Chem.*, vol. 134, p. 543.
- VOLKER, J. F. 1939 Effect of fluorine on solubility of enamel and dentin. *Proc. Soc. Exp. Biol. and Med.*, vol. 42, p. 725.
- WRAMPELMAYER, E. 1893 Ueber den Fluorgehalt der Zahne. *Zeitschrift fur analytische Chemie*, vol. 32, p. 550.
- YULE, GEORGE UDNY, AND M. G. KENDALL 1937 *Introduction to the Theory of Statistics*. Charles Griffin & Company, Limited, London, 11th ed., p. 360.

# THE ASCORBIC ACID CONTENT OF COW'S MILK DURING PREGNANCY

ARTHUR D. HOLMES, FRANCIS TRIPP, E. A. WOELFFER AND  
G. HOWARD SATTERFIELD

*Research Laboratories, The E. L. Patch Company; H. P. Hood and Sons, Boston,  
Massachusetts, and Department of Chemistry, University of  
North Carolina, Raleigh*

(Received for publication May 15, 1941)

The ascorbic acid content of cow's milk has been given considerable attention since Tillmans, Hirsch and Hirsch ('32), Harris and Ray ('35) and Bessey and King ('33) introduced reliable and rapid methods employing 2, 6-dichlorophenolindophenol for the determination of this factor in biological materials. However, a thorough survey of available literature failed to reveal any information concerning the influence of pregnancy on the amount of ascorbic acid in cow's milk. Therefore this study was conducted to determine the relationship, if any, between this physiologic state and the vitamin C content of the milk of a group of nineteen Guernsey and Holstein cows.

## METHODS

The age of the Guernseys varied from 4 to 8 years and the Holsteins from 5 to 9 years. The animals were housed in a modern dairy barn, were stall-fed, and throughout the experiment had no access to pasture. All cows were under the observation of an experienced veterinarian and were maintained for the production of certified milk under carefully controlled conditions on a Massachusetts farm. All the experimental animals were negative to the agglutination test for Brucella Infection, and mastitis was controlled by physical and laboratory tests together with approved sanitary practices.

The attendants were under constant medical supervision in order to eliminate human infections or milk-borne diseases.

The cows received a daily ration compounded from various combinations of the following ingredients: first and second grades of mixed hay, first and second grades of alfalfa hay, legume and grass silage, beet pulp, dairy ration, dairy ration with 6.7% irradiated yeast, home-mixed fitting ration with 6.7% irradiated yeast and a commercial fitting ration with irradiated yeast. The authors ('39, '40) have previously published data concerning the composition of the rations and the ascorbic acid content of their ingredients.

Samples of milk, which were assayed for their vitamin C content, were taken at bimonthly intervals from the morning milking (3:00 A.M.). The samples were collected in 30 cc. flint glass bottles which were immediately cooled, placed on ice and protected from light until assayed. The amount of ascorbic acid in the milk was determined by the usual indophenol titration method as described in a previous publication by Holmes and co-workers ('39). Since the authors ('39) found that the milk from Guernsey cows contained more vitamin C than that of Holstein cows, the results of the analyses (table 1) are reported separately for the two breeds in terms of milligrams of ascorbic acid per liter of milk. Table 1 also supplies data concerning the number of assays and the average vitamin C content of milk collected at different stages of pregnancy.

#### RESULTS

The smallest amount of vitamin C found in the milk of the Guernseys was in the ninth month of pregnancy when 17.88 mg. per liter was obtained in a single determination. This is essentially the same as 17.91 mg. per liter obtained for this group in the eighth month of pregnancy. The amount of ascorbic acid found in the milk of the Guernseys during the eighth month of pregnancy varied from 14.95 mg. per liter for cow no. 2 to 19.54 mg. per liter for cow no. 1.

The average figures for the ascorbic acid content of milk obtained for cows in the fifth month of pregnancy fell within

TABLE 1  
*Pregnancy and the ascorbic acid content of milk.*  
*All values are in milligrams of ascorbic acid per liter.*

COW NO.	MONTH OF PREGNANCY								
	1	2	3	4	5	6	7	8	9
<i>Guernseys</i>									
1	....	20.19	20.80	21.10	20.60	18.93	15.71	18.64	17.88
	....	19.50	19.70	19.10	20.31	18.70	19.69	19.54	....
2	....	22.09	23.13	20.53	16.24	18.98	19.51	14.95	....
	....	12.32	21.01	20.51	18.52	18.63	18.30	....	....
	....	22.67	....	....	....	....	....	....	....
3	19.63	20.51	20.42	21.59	19.55	18.36	19.36	18.51	....
	20.86	18.53	21.43	19.44	19.31	17.46	17.61	....	....
	....	20.48	....	....	....	....	....	....	....
4	....	....	....	....	21.37	21.17	19.55	....	....
	....	....	....	....	16.56	17.85	20.19	....	....
	....	....	....	....	....	24.30	....	....	....
5	25.48	....	....	....	....	....	....	....	....
6	22.62	21.59	21.09	17.82	....	....	....	....	....
	21.80	20.60	....	18.36	....	....	....	....	....
7	25.70	27.68	....	....	....	....	....	....	....
	27.03	22.74	....	....	....	....	....	....	....
8	23.02	21.66	21.75	22.93	....	....	....	....	....
	21.97	21.36	21.94	22.14	....	....	....	....	....
9	21.82	18.53	....	....	....	....	....	....	....
	21.16	18.52	....	....	....	....	....	....	....
10	18.40	18.19	13.97	19.88	16.86	21.08	....	....	....
	....	18.36	19.36	....	17.72	....	....	....	....
	....	....	19.33	....	....	....	....	....	....
11	21.44	21.80	22.87	20.43	17.46	....	....	....	....
	23.71	25.33	21.47	21.00	18.36	....	....	....	....
No. cows	8	9	7	7	6	5	4	3	1
No. det'ns.	14	19	15	13	12	10	8	4	1
Average	22.47	20.53	20.73	20.37	18.57	19.55	18.74	17.91	17.88
<i>Holsteins</i>									
12	16.41	15.78	13.41	9.84	16.02	....	....	....	....
	16.51	14.11	11.88	11.52	10.70	....	....	....	....
13	22.84	20.97	16.48	15.74	18.30	16.80	14.62	....	....
	18.68	....	16.71	16.41	....	16.86	....	....	....
	....	....	....	17.02	....	....	....	....	....
14	17.26	17.87	17.12	....	....	....	....	....	....
	15.43	16.83	....	....	....	....	....	....	....
15	....	....	....	....	20.12	19.41	....	....	....
	....	....	....	....	20.02	18.21	....	....	....
16	19.76	....	....	....	....	....	....	....	....
17	....	....	....	....	19.04	20.58	20.09	....	....
	....	....	....	....	19.25	20.08	....	....	....
18	14.34	15.45	16.56	19.34	18.23	16.90	17.57	15.64	....
	....	....	18.04	17.75	15.60	16.05	17.44	13.80	....
	....	....	....	18.25	....	....	....	....	....
19	16.34	16.73	18.25	12.35	19.70	16.61	16.10	....	....
	17.33	16.82	18.56	17.87	16.86	17.15	....	....	....
	15.68	....	....	....	....	....	....	....	....
No. cows	6	5	5	4	6	5	4	1	...
No. det'ns.	11	8	9	10	11	10	5	2	...
Average	17.33	16.82	16.33	15.61	17.62	17.87	17.16	14.72	...

the limits observed for the eighth month of pregnancy. The highest average vitamin C content of the group of Guernsey cows was 22.47 mg. of vitamin C per liter, for cows in the first month of pregnancy. However, cows in this first month also showed wide variations. Cow no. 10 produced milk with a vitamin C content of 18.40 mg. per liter while the milk from cow no. 7 contained 27.03 mg. per liter. The data for the Guernsey cows show that wide variations in vitamin C content of the milk occur at all stages of pregnancy. However, when the average figures are considered there is a gradual downward trend in the concentration of vitamin C as pregnancy progresses.

The data obtained for the milk from the Holstein cows show that the smallest average ascorbic acid content was 14.72 mg. of vitamin C per liter for the eighth month of pregnancy. The highest average value, 17.87 mg. per liter, was found in the milk of cows in the sixth month of pregnancy. However, this figure exceeds but slightly the amount found in the milk of the Holsteins during the first, fifth and seventh months of pregnancy. As in the case of the Guernsey cows, the milk from Holstein cows at all stages of pregnancy showed considerable variation. During the first month of pregnancy the vitamin C content of the milk varied from 14.34 mg. per liter for cow no. 18 to 22.84 mg. for cow no. 13. Likewise, during the fourth month of pregnancy the vitamin C content of the milk varied from 9.84 mg. per liter for cow no. 12 to 19.34 mg. per liter for cow no. 18.

#### SUMMARY

Milk produced by Guernsey and Holstein cows at various stages of pregnancy was assayed for its ascorbic acid content.

The highest average value was found in the milk of the Guernsey cows during the first month of pregnancy, namely, 22.47 mg. per liter. A gradual but not consistent decrease in the average vitamin C content was observed in the milk from Guernsey cows as pregnancy advanced.

The data obtained for the Holstein cows were not in agreement with those yielded by the Guernsey cows. The highest ascorbic acid content of the milk from this group of cows was found in the sixth month of pregnancy. The increase in vitamin C content of milk from the Holstein cows during the fifth, sixth and seventh months of pregnancy is unexplained.

The data obtained in this study indicate that the vitamin C content of milk from Guernsey and Holstein cows tends to decrease with the advance of pregnancy, but at all stages of pregnancy different cows show considerable variation.

#### ACKNOWLEDGMENT

The authors wish to acknowledge the very helpful assistance of Mr. A. A. Colley and Mr. E. C. Steele during the course of this study.

#### LITERATURE CITED

- BESSEY, O. A., AND C. G. KING 1933 The distribution of vitamin C in plant and animal tissues and its determination. *J. Biol. Chem.*, vol. 103, p. 687.
- HARRIS, L. J., AND S. N. RAY 1935 Diagnosis of vitamin-C subnutrition by urine analysis. *Lancet*, vol. 1, p. 71.
- HOLMES, A. D., F. TRIPP, E. A. WOELFFER AND G. H. SATTERFIELD 1939 A study of breed and seasonal variations in the ascorbic acid content of certified milk from Guernseys and Holsteins. *J. Nutrition*, vol. 17, p. 187.
- 1940 Ascorbic acid content of cow's milk at various stages of lactation. *Am. J. Dis. Child.*, vol. 60, p. 1025.
- TILLMANS, J., P. HIRSCH AND W. HIRSCH 1932 The reducing property of plant foods and its relation to vitamin C. *Ztschr. f. Untersuch. d. Lebensmitt.*, vol. 63, p. 1.



# ENERGY AND GASEOUS METABOLISM OF THE HEN AS AFFECTED BY TEMPERATURE

H. G. BAROTT AND EMMA M. PRINGLE

*Animal Nutrition Division, Bureau of Animal Industry,  
Beltsville Research Center, Beltsville, Maryland*

SEVEN FIGURES

(Received for publication February 17, 1941)

## INTRODUCTION

Constancy of body temperature of a homoiotherm is maintained even under extreme variation in environmental conditions. To maintain this constant temperature there must always be a balance between the heat production within the system and loss of heat from the system. The heat produced is supplied by the oxidation of feedstuffs or of material stored within the body. Heat is lost by radiation, convection, and as latent heat of vaporization of water. It is apparent then that environment may affect the life processes. A measurement of the metabolism is a measurement of the activity of these life processes and by studying the course of metabolism under different conditions of environment some information may be gained as to the effect of environmental conditions on the life processes.

Several investigators have studied the effect of temperature on the gaseous metabolism of the hen by use of the open circuit Haldane method. Mitchell and Haines ('27) made observations on the carbon dioxide elimination of hens at nine different environmental temperatures with 5-degree intervals between 45° and 85° F., inclusive. They concluded that there was a critical temperature (the temperature at which the metabolism is at a minimum) at 62° F. Our analysis of their

published results gives a very different temperature for the minimum metabolism. Their data were reanalyzed to obtain the carbon dioxide elimination per hour per gram of live weight of the hen and a mean value calculated for each observed temperature. These values were plotted (fig. 1) and a curve was drawn through them. This curve shows that the metabolism, as indicated by elimination of carbon dioxide, is at a minimum between 75° and 80° F.

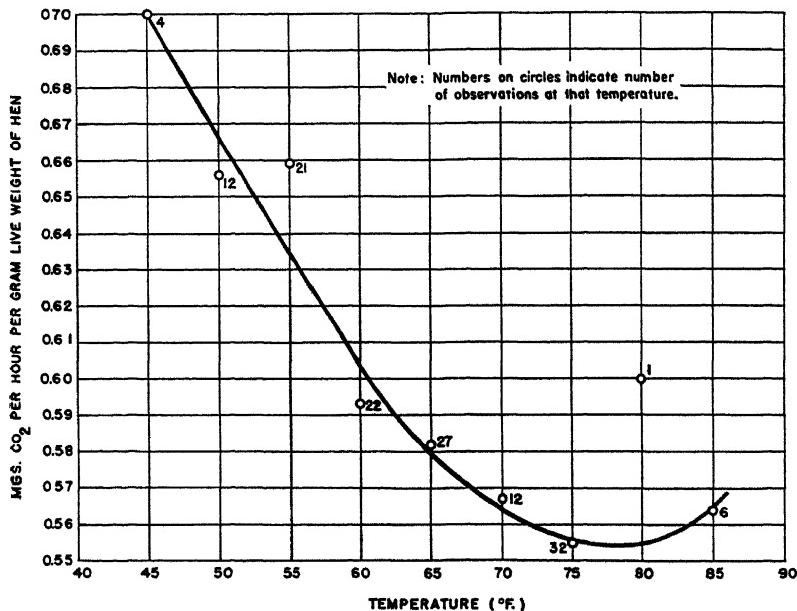


Fig. 1 Carbon dioxide elimination of hens at various temperatures. Source: Data of Mitchell, H. H., and W. T. Haines.

Terroine and Trautman ('27), using the open circuit Hal-dane method, studied the effect of temperature on the gaseous metabolism of several species of homoiotherms, among them the hen. They used birds whose weights ranged between 854 and 1,075 gm. Neither the breed nor the age of the birds is given although the article states that all animals studied were adults, but from the weight one must conclude that the hens, if adult, were undersized. The data given were for fourteen

experiments at ten different temperatures between 15° and 99° F. The results show a minimum metabolism at 78° to 80° F. The metabolic rate increased with either an increase or decrease in temperature. At 99° F. and also at 26° F. the rate was nearly twice that at 80°, while at 16° F. it was two and one-half times that at 80° where the minimum was found.

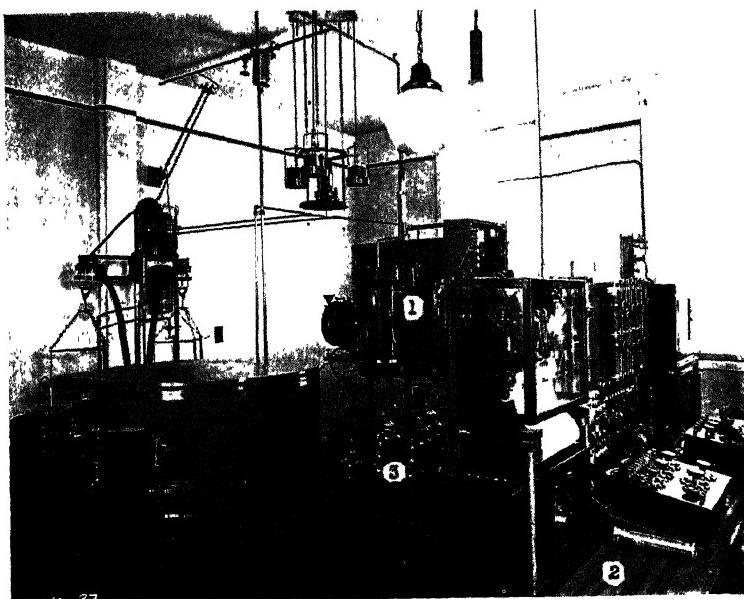


Fig. 2 Respiration calorimeters and accessory apparatus. 1, calorimeter used for this research; 2, board with instruments for measurement and control; 3, train for absorption of water and carbon dioxide.

#### PROCEDURE

The results herein reported on the effect of temperature on the metabolism of the hen were obtained by use of one of the respiration calorimeters in the Calorimetry Section of the Bureau of Animal Industry, Beltsville Research Center, Beltsville, Maryland, between April, 1938 and April, 1939. This calorimeter (fig. 2) is an instrument of precision. The construction and technique of operation are in every way identical

with the one described briefly by Barott, Byerly and Pringle ('36), and in greater detail by Barott ('37). The capacity of the system is approximately 130 l. and the flow of air to and from the chamber approximately 90 l. per minute.

In this series of experiments Rhode Island Red hens from 10 to 13 months of age were used. These hens were kept in a colony house except during an observational period and were provided with the regular laying diet. The following routine was used: A hen was brought from the house to the laboratory at daybreak. Therefore, having had no feed since the previous

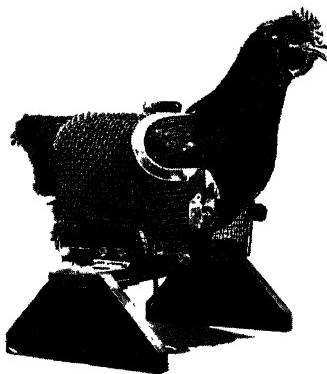


Fig. 3 Hen on perch inside cage, indicating the type of confinement during the experimental period. The semi-circular "cut-out" in front side near top is made so the wings may remain outside cage during experiments at the higher temperatures.

night, the alimentary tract was comparatively empty. The hen was weighed and placed on a wooden perch with a specially constructed copper wire cage attached (fig. 3). This cage was of such size as to confine the bird closely so that movement was kept at a minimum. The cage containing the hen was then placed in the calorimeter. A dish containing liquid petrolatum was placed back of the perch in such a position that the droppings would fall into the dish. The oil prevented the addition of water to the chamber by evaporation from the feces. The calorimeter was then closed and

sealed. The conditions that were to prevail relative to temperature and humidity were established so that the experiment could be started at 9 A.M. The total energy and gaseous metabolism were determined for one 3-hour period from 9 A.M. to noon. Thereafter, the oxygen was obtained for each 2-hour period and the water, carbon dioxide, and heat for each 4-hour period. At 8 A.M. the next day the calorimeter was opened and the hen removed and weighed. The weight of the excrement was obtained by taking the difference in weight of the dish of oil and contents before and after the experimental period.

The initial weight of the hen minus the weight of the droppings was used in computing the first value for metabolic rate. The final weight was used in computing the last value. To compute the intermediate values the difference between these two weights was prorated linearly with time.

During the experimental period the air temperature within the calorimeter was kept practically constant at a predetermined value, the relative humidity was kept between 50 and 60%, the oxygen content at 21%, and the carbon dioxide below 1%.

The experiments covered a range of temperature from 50° to 95° F. Observations were made at nineteen different temperatures within this range. One-day experiments were conducted using a different hen for each experiment. The metabolic rate for each temperature represents a mean of the results of several 1-day experiments. The number at each temperature is shown in figure 5.

#### *Energy and gaseous metabolism*

The data obtained for each hen were analyzed and the metabolism per hour per gram of live weight was calculated. These final values were plotted and a curve drawn through the plotted points in such a manner that there was an equal distribution of points on each side. Figure 4<sup>1</sup> shows a typical

<sup>1</sup>It will be noted that some points are plotted at times differing from the mean of the 2-hour or 4-hour periods, or that a point is missing. This is because the data were incomplete for that period. In some cases it was possible to combine two consecutive periods and obtain a value.

example. These data were determined at a temperature of  $84.8 \pm 0.4^\circ$  F. and are the results obtained with five different hens. The curve was extrapolated to 8 A.M. From previous data (Barott et al., '38), it is known that the maximum rate of metabolism occurs at 8 A.M.; consequently, this extrapolation can be made with small error.

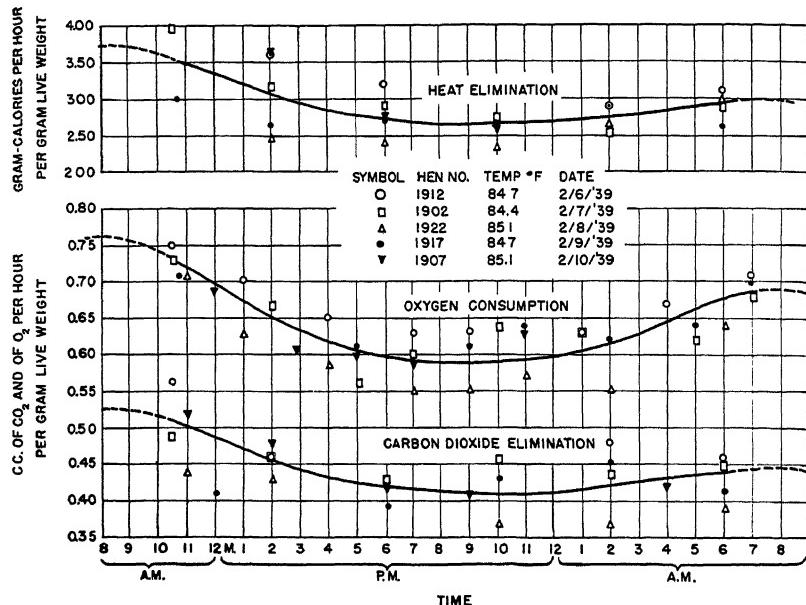


Fig. 4 Energy and gaseous metabolism of the hens.

These curves show the typical diurnal rhythm in the metabolic rate of the fowl, with a high value at 8 A.M., the rate declining until it reaches a minimum at 8 P.M., thereafter rising again until 8 A.M. The value at 8 A.M. of the second day is approximately 10% lower than that of the first day due to the fact that the birds were without feed during their stay in the calorimeter. The decrease in metabolic rate for these hens during the first day was less than 50% of that for immature birds 9 to 10 weeks of age, as previously determined (Barott et al., '38).

Charts similar to figure 4 were constructed for the results obtained at each temperature studied. These charts show the

metabolic rate during the experimental period as measured by heat and carbon dioxide elimination and oxygen consumption. By integration of the area under the curves of these charts the metabolism may be obtained for any time interval during the day.

By such integration the values were found for carbon dioxide and heat elimination and for oxygen consumption for the first 12 hours at each temperature. The mean values per hour were calculated and plotted in figure 5 and curves drawn

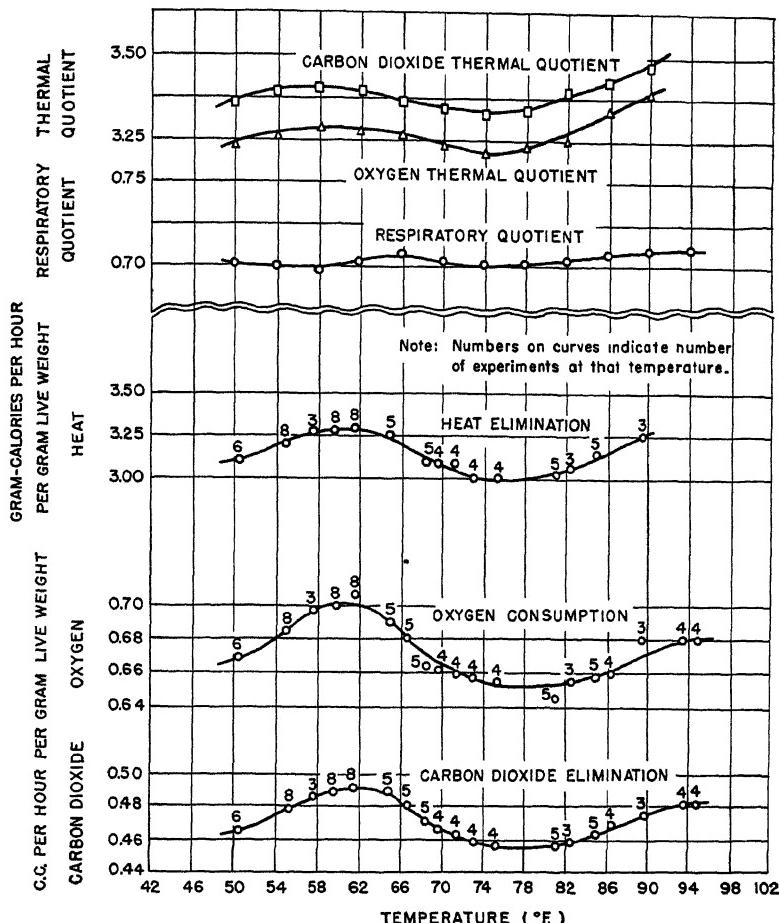


Fig. 5 Energy and gaseous metabolism (mean of the first 12 hours) respiratory quotient and carbon dioxide and oxygen thermal quotients.

through the plotted points. These values show the effect of temperature over the range from 50° to 95° F. on the metabolic rate, as indicated by these three quantities. The three curves are similar in form, each having two points of flexure, a minimum value occurring at approximately 78° F. and a maximum at approximately 61° F. The rate at 61° F. is approximately 8% higher than that at 78° F. As the temperature is increased above 78° F. the metabolic rate increases to the highest temperature (95° F.) studied. At 95° F. several of the hens died and therefore the authors did not attempt to conduct experiments at higher temperatures.

From the results obtained by Terroine and Trautman ('27) and Mitchell and Haines ('27), it was expected that the metabolic rate would increase steadily as the temperature was decreased below 60° F. Instead of doing so, the rate became lower when the temperature of 61° F. was passed and continued to decrease to 50° F. with the trend still downward and the rate approaching that at 78° F. The authors have conducted no experiments below 50° F. to date. It seems probable that the curve representing the metabolic rate has another point of flexure not many degrees below 50° F., after which the metabolic rate increases once more. If this were not true the fowl would be unable to compensate for the loss of heat due to low external temperatures and would perish. More work in the lower temperatures must be done in order to determine the metabolism of the fowl in this temperature zone.

The respiratory quotient and the oxygen and carbon dioxide thermal quotients were computed from the values for oxygen consumption and heat and carbon dioxide elimination taken from the curves in figure 5. The respiratory quotient thus obtained (for the mean 12-hour period from 8 A.M. to 8 P.M.) is approximately  $0.702 \pm 0.002$  between 50° and 80° F. The respiratory quotient becomes greater at the higher temperatures, reaching a value of 0.710 at 94° F.

The oxygen and carbon dioxide thermal quotients are respectively  $3.25 \pm 0.05$  and  $3.37 \pm 0.05$  in the temperature range

from 50° to 80° F. The thermal quotients became greater for temperatures above 75° F. as in the case of the respiratory quotient, and at 90° F. are 3.39 and 3.45, respectively.

The respiratory quotient and oxygen and carbon dioxide thermal quotients indicate that fat constituted nearly all of the material being metabolized by the hens, which probably is true since they had no feed for approximately 20 hours before 2 P.M., the mid-point of the 8 A.M. to 8 P.M. period for which the metabolic rate was determined.

#### *Water metabolism*

The discussion thus far has concerned energy and gaseous metabolism. The elimination of respiratory water was also determined in this research. The water varies to a great extent with the environment of the fowl.

Birds have no sweat glands in the skin. Therefore, when it is necessary to eliminate heat in order to control the temperature of the body, they do so by the great quantity of air they are able to inhale and exhale with each breath. This air passes through the lungs, which are comparatively small, compact, and inelastic, and into nine air sacs, which are distributed throughout much of the body and fit closely around the viscera and muscles, thus affording a method of eliminating heat by evaporation of large quantities of water from the tissue surfaces.

It was noted in our work that at the higher temperatures panting occurred and that the higher the temperature, the more pronounced was the panting. This fact led us to analyze the course of water elimination over the range of temperature studied. Figure 6 shows the rate of elimination of respiratory water, derived by computations of water elimination from our observed data. Between the temperatures of 65° F. and 75° F. the rate of elimination remains nearly constant. At temperatures below 65° F. the rate decreases at first slowly and then more rapidly as conservation of heat becomes necessary because of lower temperatures.

At temperatures higher than 75° F. the rate of elimination begins to increase and after a temperature of 80° F. is passed the amount of water thus eliminated increases very rapidly, so much so that at 90° F. over three times as much water is being eliminated from the respiratory system as was eliminated at 80° F. Above 90° F. the rate still increases slightly to 95° F. but it is apparent that the hen is approaching the point at which she can no longer increase her capacity for

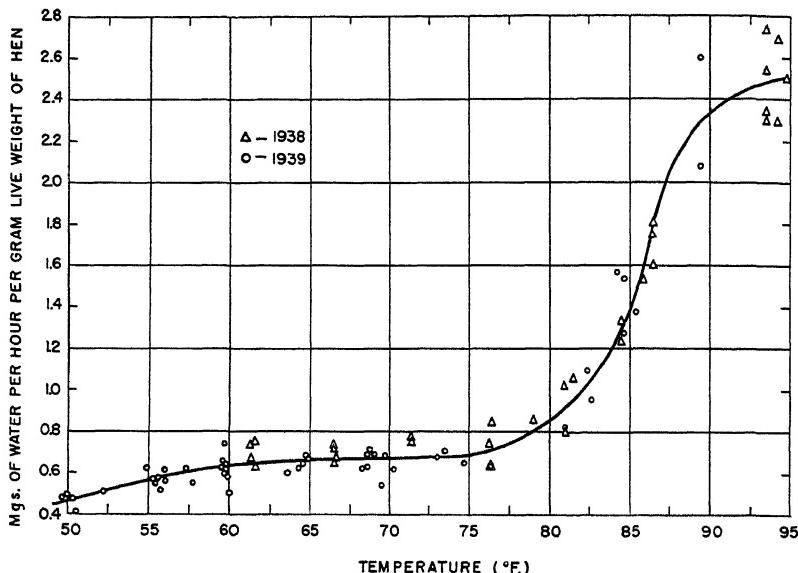


Fig. 6 Respiratory water eliminated by hens at different temperatures and at 50% relative humidity.

cooling her body by breathing, and the rate of increase in water elimination, as shown by the curve, seems to be approaching zero.

The fact that several of the hens died at a temperature of 95° F. would seem to be strong evidence that they could not longer eliminate sufficient heat, because they could no longer increase their "ventilation", and so died of heat prostration.

To prove conclusively that this was true, a series of observations were conducted with the same or similar hens at tempera-

tures between 90° F. and 100° F., and at relative humidities between 30 and 80%. It was found that hens at a given temperature would survive at the lower humidities but would die when the humidity was increased. At 90° F. all hens lived for 14 hours (the duration of the test) at all humidities below 75%. At 95° F. hens survived at humidities below 60% but perished at higher humidities; while at 100° F. they survived only at humidities of 30% or lower.

Another interesting phenomenon was noted. Some hens died at comparatively high temperatures while others subjected to the same treatment survived. Upon examination of the dead hens, in each case it was found either that the hen was very fat, the liver or internal organs were enlarged, or there was a large cluster of egg yolks in the body cavity which impeded the dilation of the air sacs. A hen without excessive fat and in such condition that its air sacs could be utilized to capacity survived with no noticeable ill effects.

The great increase in water elimination in the temperature region between 80° and 95° F. and the fall in metabolic rate at temperatures between 60° and 50° F., induced the authors to analyze the sensible and latent heat elimination over the temperature range studied.

All the data were tabulated for each observed temperature and a mean of the values for both latent heat and sensible heat at that temperature plotted in figure 7. A graph which shows the relative values for the sensible and latent heat elimination was drawn through the plotted points.

A third graph was added which shows the total heat elimination. This third graph is similar in form to that in figure 5. However, the values are somewhat lower for they represent more nearly the minimum for the 24-hour period, whereas the curve (fig. 5) represents the mean for the period from 8 A.M. to 8 P.M.

It may be noted (fig. 7) that both latent and sensible heat remain practically constant over the temperature range between 65° F. and 80° F. Between 65° and 60° F. the sensible heat tends to increase and the latent heat tends to decrease, but

the sensible heat increases much more rapidly than the latent heat decreases so that the total heat elimination is increased to compensate for the lower environmental temperatures.

One would expect the total heat elimination to continue to increase as the temperatures were lowered below 60° F. but, surprisingly, just the opposite happened. The latent heat continued to decrease, as would be expected, but the sensible heat also dropped rapidly between the temperatures of 60° and 55° F. At temperatures between 55° and 50° F. the curve

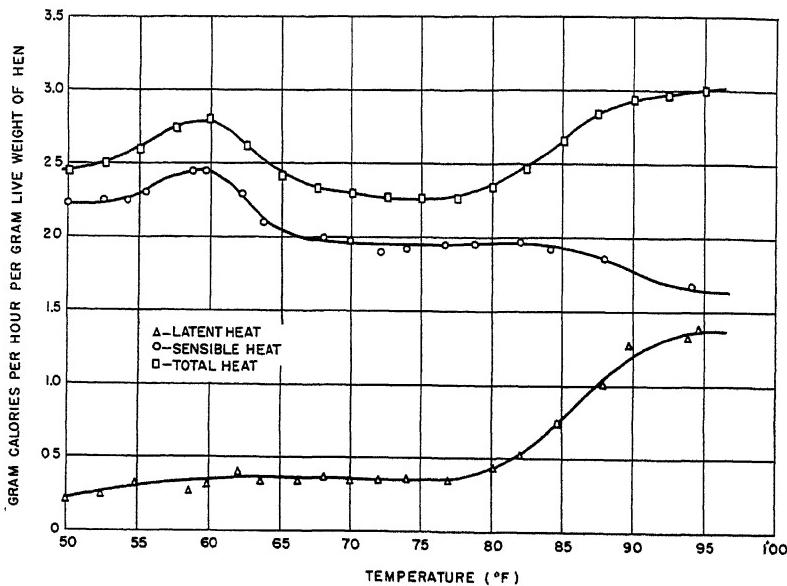


Fig. 7 Latent, sensible and total heat elimination of hens.

representing sensible heat appears to be approaching a third point of flexure. It appears probable that at temperatures lower than 50° F. the latent heat would continue to decrease and that the sensible heat would again begin to increase. However this is only a conjecture and the proof of this as well as the reasons for the drop in metabolic rate at temperatures below 60° F. must await further investigations.

The data show that in the region between 65° and 75° F. both the latent and sensible heat remain practically constant

under the experimental conditions. When the temperature is increased above 80° F. the latent heat begins to increase rapidly, indicating a large increase in water elimination in the respired air. Above 85° F. the latent heat increases rapidly to 95° F. while the sensible heat decreases in an effort to maintain the body temperature. The data indicate that at 95° F. and 60% humidity the fowl is approaching a state where the sensible heat can no longer be appreciably decreased or the latent heat increased.

#### SUMMARY AND CONCLUSIONS

The energy and gaseous metabolism of Rhode Island Red hens between 10 and 13 months of age were determined by use of one of the respiration calorimeters in the calorimetry section of the Bureau of Animal Industry, Beltsville Research Center, Beltsville, Maryland. The instrument is similar to the one described briefly by Barott ('37).

Results were obtained at nineteen environmental temperatures in the range from 50° to 95° F. Conditions other than temperature were relative humidity 60%, oxygen content 21%, and carbon dioxide content less than 1%.

The oxygen consumption was measured for each 2-hour period, and the heat, water, and carbon dioxide elimination for each 4-hour period. From the above data the respiratory quotient and the oxygen and carbon dioxide thermal quotients were computed.

The data obtained during each 24-hour period show the typical diurnal rhythm in the metabolism of the hen with a maximum value in the morning and a minimum in the evening.

The results clearly define the metabolic rate of the Rhode Island Red hen for the temperature range studied. A point of flexure occurs at 78°F. This is the temperature of minimum metabolism of the hen. The maximum metabolism occurs at 61° F., at which temperature there is also a point of flexure. The phenomenon of a decrease in metabolic rate between 60° and 50° F. should be investigated to ascertain the reason for

the decrease instead of the expected increase in metabolic rate with decrease in temperature.

The respiratory quotient was  $0.702 \pm 0.002$ . The oxygen and carbon dioxide thermal quotients were  $3.25 \pm 0.05$  and  $3.37 \pm 0.05$ , respectively, indicating a fat metabolism. This is consistent with the fact that the data were obtained approximately 20 hours after feeding.

The rate of elimination of respiratory water was approximately constant between  $65^{\circ}$  and  $75^{\circ}$  F.

The rate decreased considerably between  $60^{\circ}$  and  $50^{\circ}$  F. but the most striking change occurred between  $80^{\circ}$  and  $90^{\circ}$  F. The rate at  $90^{\circ}$  F. was three times that at  $80^{\circ}$  F. This was due to the large amount of air inhaled and exhaled for cooling at the high temperature.

#### LITERATURE CITED

- BAROTT, H. G. 1937 Effect of temperature, humidity, and other factors on hatch of hens' eggs and on energy metabolism of chick embryos. U. S. D. A. Tech. Bull. 553, pp. 1-45.
- BAROTT, H. G., T. C. BYERLY AND E. M. PRINGLE 1936 Energy and gaseous metabolism of normal and dectectomized chicks between 10 hours and 100 hours of age. J. Nutrition, vol. 11, pp. 191-210.
- BAROTT, H. G., J. C. FRITZ, E. M. PRINGLE AND H. T. TITUS 1938 Heat production and gaseous metabolism of young male chickens. J. Nutrition, vol. 15, pp. 145-167.
- MITCHELL, H. H., AND W. T. HAINES 1927 The critical temperature of the chicken. J. Agri. Res., vol. 34, pp. 549-557.
- TERROINE, E. F., AND S. TRAUTMAN 1927 Influence de la température extérieure sur la production calorique des homoéothermes et loi des surfaces. Annal de Physiol., vol. 3, pp. 422-447.

THE EFFECTS OF VARIOUS VITAMIN SUPPLE-  
MENTS AND OF WHOLE YEAST ON THE  
DIGESTION AND ABSORPTION OF  
THE CARBOHYDRATE OF A  
COMPLETE DIET<sup>1,2</sup>

R. A. RUSSELL AND E. S. NASSET

*Department of Vital Economics, University of Rochester, New York*

ONE FIGURE

(Received for publication April 10, 1941)

There are many reports in the literature linking the vitamin B-complex and the gastrointestinal tract, and in many of these studies multiple deficiencies probably existed. There is a tendency to regard thiamine as specially important in the maintenance of the normal function of the gastrointestinal tract, but experiments clearly showing such a relationship are rare. Few workers have interested themselves in the effects of supplementing what is currently considered an adequate diet with vitamins in excess of the minimum requirements for growth. There have been few studies on the adult animal, whose requirements may conceivably differ considerably from those of the young and rapidly growing one. Furthermore, most earlier vitamin work necessarily was concerned with the minimal requirements, whether for growth or maintenance, and the measure of such requirement was based largely on the rate of change or the maintenance of body weight.

<sup>1</sup> The data in this paper are taken from a thesis presented by R. A. Russell to the Division of Graduate Studies, University of Rochester, in partial fulfillment of the requirements for the degree Master of Science, 1940.

<sup>2</sup> This investigation was supported by a grant from Standard Brands, Inc.

The object of this investigation was to determine the effect, if any, of large amounts of the B-complex on the function of the normal gastrointestinal tract of the adult animal as contrasted with the growth of young animals. To this end, amounts of the B-complex well in excess of the accepted dietary requirements were fed to dogs maintained on a basal ration known to be adequate for growth, maintenance, and reproduction. The effects of such supplements on the digestion and absorption of carbohydrates and gastrointestinal motility were studied.

#### METHOD

Maydl jejunostomies were established 50 to 75 cm. from the ligament of Treitz in five healthy adult male dogs. A catheter could be inserted into the lumen of the gut whenever desired and its contents withdrawn, whereas normally they passed the stoma without leakage. These animals were maintained in excellent condition on a basal diet<sup>3</sup> which is known to fulfill the ordinary dietary requirements of both the dog and the white rat. Various supplements were fed with this diet from time to time, experimental and basal periods being alternated so that any progressive changes in gastrointestinal function could be detected.

Only after 6 to 8 days on a particular diet were the animals used for collection of intestinal contents. About 20 hours after their last feeding, the animals were placed in stalls and a no. 24 French soft-rubber catheter with several holes in it inserted into the jejunostomy. After 30 minutes of aspiration at -12 cm. of water pressure to remove residual material, the animals were fed a standard test meal consisting of 100 gm. of finely-ground basal ration moistened with about 50 ml. of water. Thus, with the exceptions noted, the performance of the upper part of the digestive tract was always tested on the basal ration regardless of what supplements the animal might be ingesting with his regular diet. In some experiments the supplements were fed with the test meal to determine whether

<sup>3</sup>Purina Dog Chow.

there was local as well as systemic conditioning of the alimentary tract. The chyme was collected at 15-minute intervals, the volume and weight of each sample recorded, and pH determinations made frequently with a glass electrode. Each sample as collected was divided into two equal parts: one was boiled immediately to stop digestion and the other was incubated under toluene at 38°C. until enzyme hydrolysis was complete (48 hours). All samples for each experiment were thus divided and pooled in two flasks, the reducing substance in each was extracted with water, proteins were precipitated with  $Zn(OH)_2$ , and the total reducing substance determined by the Shaffer-Somogyi ('33) method.

The "final emptying time" was fixed by several criteria: (1) recovery of 20 cc. or less of intestinal contents in a half-hour period, (2) absence, or at most traces, of solid food particles, (3) a pH typical of pre-feeding contents (6.0-6.5), and (4) a negative Benedict's test for reducing sugar. The collection was continued for an hour or more after the final emptying time had been determined in order to make certain that the intestine was empty.

From the analytical results and the time required for the final appearance of food, the average rates of carbohydrate digestion and absorption were calculated. The test meal contained 43.4% total carbohydrate, including 10.1% reducing substance. In calculating the rates of digestion it was assumed that the 10.1% of reducing substance was absorbed first and required no digestion. This amount subtracted from the sum of absorbed and recovered reducing substance gave the amount digested. This accounts for the apparently anomalous situation that the animal absorbed more than he digested (table 1).

The  $t$  test as described by Fisher ('34) for small samples was used in calculating the significance of differences observed between the rates of digestion and absorption on the several experimental diets, and the rates on the unsupplemented basal diet.

## RESULTS

Table 1 summarizes the results obtained with five dogs.

TABLE 1

*Carbohydrate digestion and absorption as affected by fresh yeast.*

DOG NO.	CARBOHYDRATE ABSORBED PER HOUR			CARBOHYDRATE DIGESTED PER HOUR		
	Basal ration	Basal ration plus yeast	Change	Basal ration	Basal ration plus yeast	Change
	gm.	gm.	%	gm.	gm.	%
1	5.75 (14) <sup>1</sup>	6.87 (7)	+19	5.09	5.94	+16
2	7.03 (4)	7.81 (4)	+11	5.70	6.03	+ 6
3	6.98 (3)	7.06 (3)	+ 1	5.88	5.72	- 3
4	6.78 (5)	7.67 (3)	+13	5.58	6.07	+ 9
5	10.24 (4)	11.46 (2)	+12	8.22	8.99	+ 9

<sup>1</sup> Number of experiments averaged in parentheses.

The first supplement used in this study was fresh commercial foil-wrapped yeast, which was fed at the level of two cakes (about 26 gm.) per day. It was found that feeding yeast with the test meal had a pronounced effect on gastrointestinal motility, producing larger initial volumes and a shorter final emptying time (fig. 1). Comparison with the

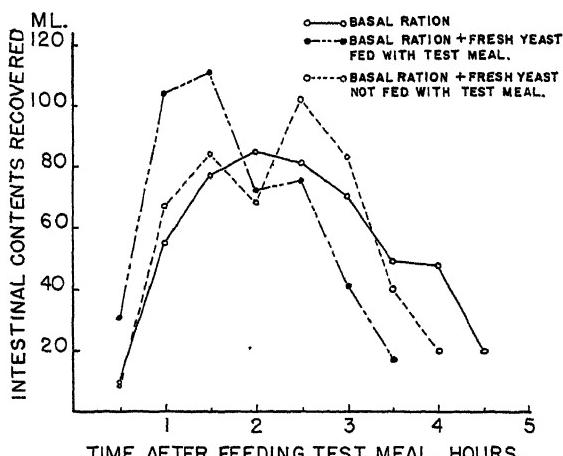


Fig. 1 Test meal recoveries from jejunostomy in dog 1. Basal ration: 22 experiments. Basal ration and fresh yeast—yeast fed with test meal: 7 experiments. Basal ration and fresh yeast—yeast not fed with test meal: 7 experiments.

curve for a series of experiments where yeast was not fed with the test meal, although the animal was on a yeast regime, reveals that this effect is largely local in nature, owing to the presence of live yeast in the gastrointestinal tract. The use of two cakes of fresh yeast with the test meal causes fermentation and makes carbohydrate analyses meaningless. It was found, however, that even without the presence of live yeast in the test meal, the rates of digestion and absorption were increased in four of the five dogs (table 1), although the effects on motility were greatly reduced. This suggests that fresh yeast acts in at least two ways on the gastrointestinal tract, namely, its actual presence has a pronounced effect on motility, and its regular feeding prior to experiment conditions the alimentary tract so that digestion and absorption are more rapid.

With the observation of these effects experiments were begun to determine if the crystalline vitamins<sup>4</sup> then available were the causative agents. Supplements containing the yeast equivalents of crystalline thiamine (1.3 mg.), riboflavin (0.6 mg.), nicotinic acid (1.0 mg.) and pyridoxine (0.4 mg.), together with highly concentrated preparations of vitamins A and D, were administered daily with the basal ration instead of the yeast. It was found that such supplements whether given with the test meal or not had no effect either on gastrointestinal motility or on the rates of digestion and absorption. Five and ten times these amounts were similarly without effect, showing conclusively that this combination of vitamins was not responsible for the yeast effects. This indicates also that feeding excessive amounts of these factors, over and above the ordinary dietary requirements, has no effect on the gastrointestinal functions as studied in this investigation.

The baker's yeast was then dried and tested on the same animals at an intake equivalent to two cakes of fresh yeast per day. It was found to produce about the same increase in the rates of digestion and absorption, without influencing motility. The 50% alcohol-soluble fraction of dried yeast con-

<sup>4</sup>Kindly supplied by Merck and Company, Inc.

tained most of the active principles. Even when fed with the test meal, neither the dried yeast nor the extract gave the effects on volumes that were observed when fresh yeast was fed.

Shortly before the conclusion of this work crystalline d-l-calcium pantothenate became available and 2 mg. per day were added to a vitamin supplement of thiamine, riboflavin, nicotinic acid, and pyridoxine, which previously had been without effect on the gastrointestinal tract. The addition of calcium pantothenate was accompanied by a marked increase in

TABLE 2  
Summary: dog 1. Carbohydrate digestion and absorption on several diets.

DIET		CARBOHYDRATE ABSORBED PER HOUR				CARBOHYDRATE DIGESTED PER HOUR			
No.	Description	Grams	% above basal	t <sup>1</sup>	P <sup>2</sup>	Grams	% above basal	t <sup>1</sup>	P <sup>2</sup>
1	Unsupplemented basal ration (14) <sup>3</sup>	5.75				5.09			
2	No. 1 plus fresh yeast (7)	6.87	19	2.34	0.03-0.04	5.94	16	2.20	0.04-0.05
3	No. 1 plus dried yeast (3)	7.04	22	3.12	0.01	5.82	14	2.16	0.04-0.05
4	No. 1 plus yeast extract (3)	6.91	20	2.79	0.01-0.02	6.27	23	2.92	0.01-0.02
5	No. 1 plus thiamine, riboflavin, pyridoxine & nicotinic acid (5)	5.84	1	0.23	0.8-0.9	5.01	-2	0.25	0.8-0.9
6	No. 5 plus d-l-calcium pantothenate (3)	8.67	51	6.64	0.01	6.95	37	5.24	less than 0.01

<sup>1</sup> When compared with unsupplemented basal ration.

<sup>2</sup> Probability that difference occurred by chance.

<sup>3</sup> Number of experiments averaged in parentheses.

the rates of digestion (51%) and absorption (37%) of carbohydrate in the three experiments in which it was used. These experiments strongly suggest that pantothenic acid is responsible for at least a part of the efficacy of whole yeast.

In table 2 is given a summary of the rates of digestion and absorption of dog 1 on the several experimental diets de-

scribed, together with *t* values and the probabilities of a chance deviation of the results on supplemented diets from the control diet.

On all of the diets used a consistent inverse relationship between volume and pH was observed. Small samples such as were recovered at the beginning and end of alimentation showed a pH range of 6.0–6.5 or higher, while the larger samples recovered at the height of the process ranged from 4.5 to 6.0. Rarely did the pH of the intestinal contents exceed 7.0.

#### DISCUSSION

The evidence shows that digestion and absorption of carbohydrate may be hastened by adding whole B-complex in the form of yeast to a diet currently considered adequate in all respects. It is generally conceded that adding an excess of certain individual members of the B-complex to a complete diet has no effect. The present experiments on the use of thiamine, riboflavin, nicotinic acid, and pyridoxine confirm this idea with respect to the gastrointestinal tract. However, the numerous experiments with whole yeast and a few experiments with pantothenic acid suggest that what currently passes for a complete diet may not, in fact, be adequate for optimal function in all respects.

It is known that yeast extracts excite the secretory cells of the small intestine and stomach (Boldyreff, '31; Bourns, Nasset and Hettig, '36; Williams, Cox and Nash, '40) and this fact may account in part for the accelerated digestion and absorption noted in the present work.

#### SUMMARY

The effects of various vitamin supplements and of whole yeast on digestion and absorption were studied in dogs with jejunostomies.

It was found that fresh yeast has a distinctly stimulatory effect on gastrointestinal motility, which is largely local in nature, and probably due to the presence of live yeast in the gastrointestinal tract.

The increased motility is often but not always accompanied by increased rates of digestion and absorption of carbohydrate.

Dried yeast and a 50% alcohol-soluble extract of this material have no effect on motility but increase the rates of digestion and absorption by about 20%.

Supplementing an already adequate basal diet with additional amounts of thiamine, riboflavin, nicotinic acid, and pyridoxine has no effect on gastrointestinal tract measurable by the technic employed.

Evidence is presented indicating that crystalline pantothenic acid may be responsible for at least part of the yeast effect.

Mr. John Lambooy was kind enough to prepare the 50% alcohol-soluble yeast extract.

#### LITERATURE CITED

- BOLDYREFF, E. B. 1931 Effect of yeast extract on intestinal secretion. *J. Mich. State Med. Soc.*, vol. 30, p. 271.
- BOURNS, T. L., E. S. NASSET AND R. A. HETTIG 1936 On the adaptive secretion of the glands of the jejunum. *Am. J. Physiol.*, vol. 116, p. 563.
- FISHER, R. A. 1934 *Statistical Methods for Research Workers*. Oliver and Boyd, London.
- SHAFFER, P. A., AND M. SOMOGYI 1933 Copper-iodometric reagents for sugar determinations. *J. Biol. Chem.*, vol. 100, p. 695.
- WILLIAMS, E. F., JR., W. W. COX AND T. P. NASH, JR. 1940 Effects of injection of extract of yeast on gastric secretion in dogs. *Am. J. Physiol.*, vol. 131, p. 378.

# FURTHER OBSERVATIONS ON RIBOFLAVIN AS A FOOD FACTOR IN ECONOMY OF FOOD UTILIZATION<sup>1</sup>

BARNETT SURE

*Department of Agricultural Chemistry, University of Arkansas, Fayetteville*

ONE FIGURE

(Received for publication April 5, 1941)

In a recent communication (Sure and Dichek, '41) it was demonstrated that riboflavin produces a pronounced effect on economy of food utilization for synthesis of body tissues and that the increases in body gains were derived mainly from fats and to a lesser extent from proteins. The data submitted represented results of studies on thirty-three pairs of animals on two types of diets, the control animals having been restricted to the same amount of food consumed by the litter mate riboflavin deficient rats. Nothing was mentioned in the recent paper concerning the pathological symptoms and the plane of nutrition of the riboflavin deficient animals during various stages of this avitaminosis, and it was not clear whether, as in thiamine deficiency, marked anorexia complicated the final collapse from riboflavin deficiency.

In this study evidence is submitted to the effect that, unlike in thiamine deficiency, the final collapse in riboflavin deficiency may be associated with either moderate reduction, no reduction, or on the contrary even greater food intake in final stages compared with early stages of riboflavin deficiency. In fact, it would seem that some riboflavin deficient animals in the

<sup>1</sup> Research paper no. 675, Journal Series, University of Arkansas. This paper is XXII in the series of AVITAMINOSIS. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

later stages of deficiency behave very much like diabetics, exhibiting abnormal hunger, thus craving compensation for inadequate utilization of foods.

This study was carried out with ten pairs of male rats, according to technique previously described (Sure and Dichek, '41). Five pairs were given our diet 13 of the following percentage composition: Casein,<sup>2</sup> 18; salts,<sup>3</sup> 4; butterfat, 10; cod liver oil, 2; and dextrin, 66. The dextrin carried an 80% alcoholic extract of 25 gm. rice polishings in a 100 gm. ration, as a source of all the components of the vitamin B complex with the exception of riboflavin. Each animal also received daily 10 µg. thiamine and 10 µg. pyridoxine, and the control animals received in addition 20 µg. riboflavin daily.

Another five pairs of male rats were given a diet with the following percentage composition: Casein (vitamin-free),<sup>4</sup> 18; agar-agar, 2; nicotinic acid, 0.05; salts,<sup>5</sup> 4; butterfat, 10; and dextrin, 65.95. This ration was supplemented with daily amounts to each animal of 20 µg. thiamine, 20 µg. pyridoxine, 6 mg. choline chloride, 100 µg. calcium pantothenate, and 150 mg. of rice polish factor II (Supplee, Bender and Kahlenberg, '40). The control animals also received 20 µg. riboflavin daily. As a source of vitamins A and D, 2 drops of halibut liver oil was given once weekly to each animal.

The results of this investigation are submitted in table 1 and in figure 1.

In table 1 additional evidence is submitted showing that riboflavin produces a marked influence in economy of food utilization. Whereas, during an average experimental period of 125 days, the average gain in body weight of the riboflavin deficient animals was 6.1 gm. per rat, during the same period of time on the same amounts of the same ration the litter mate controls, which had received 20 µg. riboflavin daily, gained 61.3 gm. per animal. We have since determined that

<sup>2</sup> Thoroughly washed with acidulated water and dilute ethyl alcohol.

<sup>3</sup> Phillips, P. H., and E. B. Hart. The effect of organic dietary constituents on fluorine toxicosis in the rat. *J. Biol. Chem.*, vol. 109, p. 658, 1935.

<sup>4</sup> Supplied by the Borden Company, New York, under the trade name "Labco."

<sup>5</sup> See footnote 3.

optimum results are obtained in riboflavin studies of economy of food utilization when animals are taken at initial weight of 55 to 70 gm. If the recommendations of Day, Darby and Cosgrove ('38) are followed, in starting with animals weighing

TABLE 1  
*Riboflavin as a food factor in economy of food utilization*

PAIR NUMBER	RATION NUMBER	PERIOD OF EXPERIMENTATION	INITIAL WEIGHT	FINAL WEIGHT	CHANGE IN WEIGHT	AVERAGE DAILY FOOD INTAKE DURING FIRST 10 DAYS OF EXPERIMENTAL PERIOD	AVERAGE DAILY FOOD INTAKE DURING LAST 10 DAYS OF EXPERIMENTAL PERIOD
		days	gm.	gm.	gm.	gm.	gm.
1-P <sup>1</sup>	13	115	72	67	-5	6.36	8.65
	RC		80	161	+81		
2-P <sup>1</sup>	13	107	72	60	-12	6.47	6.09
	RC		80	100	+20		
3-P <sup>1</sup>	13	152	72	70	-2	7.40	5.38
	RC		71	157	+86		
4-P <sup>1</sup>	13	80	72	49	-23	4.98	4.08
	RC		84	82	-2		
5-P	13	161	61	74	+13	5.99	4.90
	RC		61	130	+69		
6-P <sup>1</sup>	14	148	70	88	+18	7.37	5.23
	RC		65	140	+75		
7-P <sup>1</sup>	14	106	77	58	-19	6.06	4.48
	RC		70	92	+22		
8-P	14	115	64	130	+66	8.07	7.79
	RC		63	171	+108		
9-P	14	115	70	100	+30	7.40	4.90
	RC		64	116	+52		
10-P <sup>1</sup>	14	150	59	54	-5	6.84	6.93
	RC		60	162	+102		

<sup>1</sup>P = pathological or riboflavin deficient animal; RC = restricted control. In every pair except 5, 8 and 9 the period of experimentation was determined by the death of animal P.

from 30 to 45 gm., in order to insure the production of cataracts, such small animals take many weeks to adjust themselves to the new experimental rations, during which periods they consume very small amounts of food, so that the results in economy of food utilization are not as clear-cut as when

larger animals are selected at the beginning of the experiments. For this reason, the data submitted in this paper show riboflavin as producing much greater economy in food utilization than the findings reported in our recent paper (Sure and Dichek, '41).

We have noted that during the later stages of deficiency, even near the point of collapse, the riboflavin deficient animals are ingesting on the average very nearly the same amounts of food as they did during the first 10 days on the experiments when the daily food intake is generally at its optimum. Therefore, when an average of food consumption is taken for the first 10 days and the last 10 days of the experiments of animals which ultimately died from riboflavin deficiency, it is possible to determine to what extent, if any, anorexia has been a complicating factor in the terminal stages of this deficiency disease.

Of the ten pairs represented in this study, seven pathological animals died and the changes in the average daily food intake during the last 10 days compared with the first 10 days of the experimental periods were as follows: +36%, -6%, -27%, -18%, -29%, -26%, and +1%.

The average daily food intake during the last 10 days compared with that for the first 10 days of the experimental period for seven riboflavin deficient animals that died, results of which have recently been reported (Sure and Dichek, '41) are as follows: +12%, -21%, -5%, +4%, -6%, and +45%. Averaging all these figures for thirteen animals that succumbed from riboflavin avitaminosis we obtain a reduction of only 3%. The pathological animals of pairs 1 and 10 were actually eating more food daily preceding their total collapse than at any time during the entire experimental periods. The riboflavin deficient rat in pair no. 10 consumed 7 gm. during the 24-hour period preceding his death and was observed eating just 1 hour before he died. This is an entirely different picture from that encountered in thiamine deficiency, which produces in the last stages an inanition so pronounced as to approximate starvation (Karr, '20; Cowgill, '21; Cowgill and associates, '25 and Sure, '28). We also frequently en-

counter pronounced anorexia in terminal stages of vitamin A deficiency. This certainly does not take place in riboflavin deficiency. Since the anorexia referred to in our recent communication (Sure and Dichek, '41) was cured with the daily administration of 10 µg. pyridoxine to each animal, it is most probable that we were dealing with a pyridoxine deficiency rather than a lack of riboflavin.

The pathological symptoms noted in our riboflavin deficient animals are as follows: Alopecia, frequently accompanied by

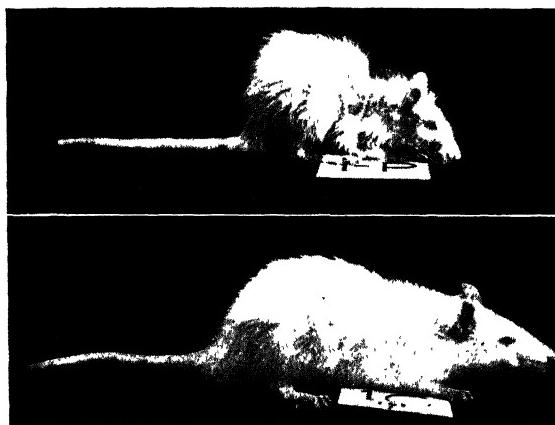


Fig. 1 Rats of pair no. 10, 1-P being riboflavin deficient and 1-C being normal. Both received the same amount of food of the same ration, but 1-C was given in addition 20 µg. riboflavin daily. Photographs taken 1 day before the 1-P animal died. Weight of 1-P, 54 gm. Weight of 1-C, 162 gm. The 1-P was eating about 7 gm. daily during the last few days of his life but was unable to utilize his food which resulted in his death. On the same daily food intake 1-C, receiving riboflavin, was still growing. Note the rough coat, alopecia, conjunctivitis and senility of the 1-P animal.

dermatitis and ulcerations in the denuded areas, rough hair, conjunctivitis, and keratitis, occasionally associated with a discharge of a granular exudate, and in the terminal stages, muscular incoordination evidenced by the animals walking on their tip-toes and their inability to balance properly on the hind legs. Premature senility is apparent in all cases of advanced stages of riboflavin deficiency. A typical illustration is submitted in figure 1.

The question arises as to what causes animals to collapse in the terminal stages of riboflavin deficiency when they are at the optimum plane of nutrition commensurate with the character of diet at their disposal. The findings of (1) Ochoa and Rossiter ('39) that there is a decrease in the total riboflavin-adenine dinucleotide content of the heart and liver, (2) Axelrod, Sober and Elvehjem ('40) that there is a decrease in the amino acid oxidase in liver and kidney in riboflavin avitaminosis, and (3) particularly the demonstration of Warburg and Christian ('38) that riboflavin is a constituent of the "yellow enzyme," would indicate that this component of the vitamin B complex is concerned with oxidative enzymic processes essential for normal metabolism. A deficiency of riboflavin may then result in a waste of products of metabolism with resultant final collapse of the animal organism.

#### SUMMARY

This study was carried out with ten pairs of male rats by the paired feeding method of experimentation on two types of diets. The average gain in body weight of the riboflavin deficient animals, during an average experimental period of 125 days, was 6.1 gm. per rat, while during the same period of time on the same amounts of the same ration the litter mate controls, by virtue of having received 20  $\mu$ g. riboflavin daily, gained 61.3 gm. per animal. To demonstrate the greatest influence that riboflavin exerts in economy of food utilization, experiments should be started on animals weighing 55 to 70 gm. rather than 30 to 45 gm. Experimental evidence has been submitted showing that, unlike in thiamine deficiency which is accompanied with a progressive inanition ultimately approaching starvation, in riboflavin deficiency there is no complicating marked anorexia in the terminal stages of the disease. There may be a moderate reduction, no reduction, or even an increased food intake in the last stages of riboflavin avitaminosis.

The riboflavin deficient rats show alopecia, dermatitis at the denuded areas of the skin, rough hair, conjunctivitis, keratitis, and premature senility.

Since riboflavin is concerned with oxidative-enzymic processes essential for normal metabolism, it is suggested that a deficiency of this vitamin may produce a waste of products of metabolism which ultimately results in collapse of the animal organism.

#### LITERATURE CITED

- AXELROD, A. E., H. A. SOBER AND C. A. ELVEHJEM 1940 The d-amino acid oxidase content of rat tissues in riboflavin deficiency. *J. Biol. Chem.*, vol. 134, p. 749.
- DAY, P. L., W. J. DARBY AND K. W. COSGROVE 1938 The arrest of nutritional cataract by the use of riboflavin. *J. Nutrition*, vol. 15, p. 83.
- COWGILL, G. R. 1921 A contribution to the study of the relation between vitamin B and the nutrition of the dog. *Am. J. Physiol.*, vol. 57, p. 420.
- COWGILL, G. R., H. J. DEUEL, JR., AND A. H. SMITH 1925 Quantitative aspects of the relation between vitamin B and appetite in the dog. *Am. J. Physiol.*, vol. 73, p. 106.
- KARR, W. G. 1920 Some effect of water-soluble vitamine upon nutrition. *J. Biol. Chem.*, vol. 44, p. 255.
- OCHOA, S., AND R. A. ROSSITER 1939 Flavin-adenine-dinucleotide in rat tissues. *Biochem. J.*, vol. 33, p. 2008.
- SUPPLEE, G., C. R. BENDER AND O. J. KAHLENBERG 1940 The effect of complementing factors on the quantitative response and specificity of vitamin B<sub>6</sub>. *J. Nutrition*, vol. 20, p. 109.
- SURE, B. 1928 A detailed study of the role of vitamin B<sub>1</sub> in anorexia in the albino rat. *J. Nutrition*, vol. 1, p. 49.
- SURE, B., AND M. DICHEK 1941 Riboflavin as a factor in economy of food utilization. *J. Nutrition*, vol. 21, p. 453.
- WARBURG, O., AND W. CHRISTIAN 1938 Bemerkung über gelbe Fermente. *Biochem. Z.*, Bd. 298, S. 368.



## THE PRODUCTION OF HIGH VITAMIN A MILK BY DIET<sup>1</sup>

HARRY J. DEUEL, JR., NELLIE HALLIDAY, LOIS F. HALLMAN,  
CORNELIA JOHNSTON AND ALBERT J. MILLER

*Department of Biochemistry, University of Southern California School of Medicine,  
Los Angeles*

(Received for publication April 21, 1941)

It has long been recognized that the carotene content of milk shows a marked seasonal variation coincident with the periods when green feeds are available. Baumann and Steenbock ('33) demonstrated that the carotene content of summer butter was increased as much as 400% over that of winter butter. These investigators found that the increase in carotene was accompanied by a concomitant but less marked rise in the content of vitamin A, the rise in the latter case being only approximately 200%.

Because of the relative difficulty in absorption and metabolism of beta carotene, it has been impossible to increase the carotene or vitamin A in the milk further even by the administration of large quantities of carotene (Baumann, Steenbock, Beeson and Rupel, '34). The attempt to increase the vitamin A content of milk by the administration of supplements of cod liver oil has also proved impractical because of the toxic symptoms produced when large quantities of cod liver oil are fed. The most pronounced effect first described by Golding et al. ('26), and confirmed by others (Petersen, '32; Hart, Hadley and Humphrey, '32; McCay and Maynard, '35; McCay, Paul and Maynard, '38), is the prompt lowering in the level of

<sup>1</sup> This work was assisted by a research grant from California Packing Corporation. Dr. J. P. Nutall and Dr. C. M. Bonyngé of the Los Angeles Certified Milk Commission assisted in the planning of the experiments. The tests on the cows were made with the cooperation of the Adohr Milk Farms, Los Angeles

butterfat in the milk. Golding ('28) reported that the non-saponifiable fraction of the cod liver oil fails to produce this effect, a result corroborated by McCay and Maynard ('35). The latter investigators found that the triglyceride portion is responsible for the decrease in milk fat while shark liver oil and salmon oil were found to be free from this property. After hydrogenation the toxic effect of cod liver oil is largely lost (McCay, Paul and Maynard, '38). Golding and Zilva ('28) reported that more than 56 gm. of cod liver oil per cow was necessary to produce this effect while the doses employed by McCay and Maynard were considerably higher ( $\frac{1}{2}$  cc. per kilo). Hilditch and Thompson ('36) found that the administration of cod liver oil increased the secretion of the unsaturated fatty acid of the C 20-22 series. However, salmon oil which is also rich in these acids fails to cause the decrease in milk fat with the constancy of cod liver oil.

The recent availability on the market in large amounts of shark liver oil which is far richer in vitamin A than cod liver oil and which contains comparatively small amounts of vitamin D has prompted the study of its effect on the nutrition of cows. The present report gives the results of such administration on the composition of the milk in vitamin A while another paper (Deuel, '41) describes the effect of this supplement on the milk and butterfat production.

#### EXPERIMENTAL

The experiments were carried out on Guernsey cows of a large certified dairy. The animals received a diet which consisted of approximately 5 pounds of a mixture composed of barley, bran, cottonseed press meal, copra, grape residue, and yeast (70 gm.) which was fed twice daily, and of alfalfa fed ad libitum. During all except the last part of the test, when baled alfalfa was used (after December 1st), the alfalfa was fresh-cut. However, since both the control cows (which received only this basal diet) and the experimental cows (which were fed also the vitamin A supplement) were given the

same food, the differences in the vitamin A secretion must be ascribed to the supplement.

The first series of tests were carried out on twelve cows from August 5, to December 23, 1940, although several of the experiments were continued until February, 1941. After a preliminary basal period of 5 weeks during which weekly determinations were made of the carotene and vitamin A content of the milk, shark liver oil<sup>2</sup> was administered in two equally divided doses of 30 cc. daily (approximately 700,000 I.U.) to six of the cows (supplement groups I and II) while the remaining animals (control group) continued on the basal diet without supplement throughout the experiment. Milk samples were collected from the control and supplement groups at 2, 3 and 9 weeks during this period. At the beginning of the eleventh week during the experimental period, the vitamin A supplement was doubled in the case of three cows (supplement group II) to 60 cc. daily containing approximately 1,400,000 I.U. of vitamin A. The remaining three cows in supplement group I continued on the lower dose. Samples were obtained during the fifteenth week of the experiment as well as during the nineteenth and twenty-third weeks in several cases.

A second series of tests (supplement group III) was made on six cows from November 11, 1940, to January 27, 1941. After a preliminary basal period of 1 week, these cows were given 10 cc. of shark liver oil daily (233,000 I.U.) for 6 weeks after which the supplement was doubled to 20 cc. (467,000 I.U.). Samples were collected during the second and fifth weeks on the lower dose and the fourth week after initiation of the higher dosage.

Records of the weight of the milk produced were maintained throughout these experiments. When the samples were collected periodically for the analysis of carotene, vitamin A, and butterfat, approximately 50 cc. aliquots were saved from

<sup>2</sup>Shark liver oil having a vitamin A potency of 25,000 U.S.P. units per gram was furnished by California Packing Corporation in the form of its Dairy Grade "Anim-A" Brand Oil.

each milking and kept in the refrigerator until the end of the week; the fourteen aliquots were mixed, a new aliquot taken for fat analysis and the balance churned in a small motor-driven Dazey churn after souring overnight with a culture of lactic acid bacilli. The induced souring of the milk prior to churning did not result in any demonstrable effect upon the carotene or vitamin A content of the milk. After collecting the butter by pouring through cheesecloth, it was washed to remove the casein, melted in the incubator and allowed to settle out after which it was carefully decanted through anhydrous  $\text{Na}_2\text{SO}_4$  on a filter to remove any water in the fat layer.

Carotene was determined by the method of Koehn and Sherman ('40) making use of the Leitz-Mass colorimeter employing a filter with maximum transmission at 440 m. $\mu$  in place of the Evelyn colorimeter used by the other investigators. Although a preliminary removal of the xanthophyll by extraction with 85, 90 and 92% methyl alcohol was not employed in the individual analyses, the amount of this pigment was found to be appreciable; the corrections found in the determinations made later on composite samples were used for recalculation of the earlier values since it has been shown that xanthophyll is not capable of transformation to vitamin A (Kline, Schultze and Hart, '32).

Vitamin A was determined on the unsaponifiable fraction making use of a Bills-Wallenmeyer electronic photometer which had previously been calibrated with the unsaponifiable fraction of reference cod liver oil. Corrections were made for the absorption due to carotene.

#### RESULTS

Typical experiments for the first series of tests are given in table 1 while table 2 summarizes the average results obtained in this group of experiments. Table 3 records the data on the fat content of the milk while table 4 gives the results of the second series of tests.

## DISCUSSION

When a sufficiently large excess of vitamin A is administered to cows, an increase in the vitamin A content of their milk results. Although the feeding of 10 or even 20 cc. of shark liver oil containing 233,000 and 467,000 I.U. of vitamin A respectively to cows daily did not result in an increased level of vitamin A in the milk, a uniform and marked rise followed the dose of 30 cc. containing 700,000 I.U. A more pronounced increase in the quantity of vitamin A eliminated resulted when the threshold level was further exceeded by doubling the dosage to 60 cc. of shark liver oil containing

TABLE 1

*The beta carotene and vitamin A per gram of butterfat in the milk of two Guernsey cows during a basal period followed by an experimental period when the basal diet was continued (control cow 5) or when it was supplemented with shark liver oil (cow 10).*

SAMPLE FROM WEEK STARTING	BETA CAROTENE IN MICROGRAMS		VITAMIN A IN I.U. <sup>1</sup>		TOTAL VITAMIN A IN I.U.	
	Cow 5	Cow 10	Cow 5	Cow 10	Cow 5	Cow 10
Basal period						
1940						
8-5	10.8	8.9	25	24	34	32
8-12	7.7	11.5	49	19	49	29
8-19	9.1	9.8	29	20	37	29
8-26	12.3	7.1	47	34	57	40
Average	10.0	9.3	38	24	44	32
Experimental period <sup>2</sup>						
9-16	14.6	10.8	37	67	49	76
9-23	16.2	10.8	19	66	32	75
11-4	13.3	7.4	21	63	32	69
12-16	12.5	4.6	..	123	..	127
1-12-41 <sup>3</sup>	5.7	2.5	26	170	31	172
2-12-41 <sup>3</sup>	....	3.0	..	168	..	170

<sup>1</sup> International Units.

<sup>2</sup> 700,000 I.U. of vitamin A administered to cow 10 daily after September 9th and 1,400,000 units daily after November 18th. No vitamin A supplement was given to control cow 5.

<sup>3</sup> One-day sample only.

approximately 1,400,000 I.U. daily. After 3 weeks on this higher level, the average vitamin A content of the butterfat had increased to 113 I.U. per gram while after 7 and 11 weeks values of 170 I.U. were noted with one animal. It would seem probable that such an increase might become greater with a still higher intake of vitamin A.

Although the vitamin A level is based largely on spectrophotometric determinations, a bioassay carried out on the butterfat of a control cow (no. 5) and an oil-fed cow (no. 10)

TABLE 2

*Summary table showing average beta carotene and vitamin A per gram of butterfat in the milk of Guernsey cows during a basal period followed by an experimental period when the basal diet was continued (control cows) or when it was supplemented with shark liver oil (supplement group). Six cows in each group.*

SAMPLE FROM WEEK STARTING	BETA CAROTENE IN MICROGRAMS		VITAMIN A IN I.U. <sup>1</sup>		TOTAL VITAMIN A IN I.U.	
	Control cows	Supplement- fed cows	Control cows	Supplement- fed cows	Control cows	Supplement- fed cows
Basal period						
1940						
8-5	10.8	9.9	34	34	43	42
8-12 <sup>2</sup>	12.5	12.0	33	24	43	34
8-19	12.2	13.1	25	24	36	33
8-26	10.9	9.9	41	39	50	48
Average	11.6	11.2	33	40	43	39
Experimental period <sup>3</sup>						
9-16	13.4	12.4	30	59	41	69
9-23	14.2	12.0	23	52	35	62
11-4	14.4	8.3	24	57	36	64
12-16 <sup>4</sup>	13.2	7.5(I)	44	65	53	72
12-16		6.8(II)		107		113

<sup>1</sup> International Units.

<sup>2</sup> Five experiments only.

<sup>3</sup> 700,000 I.U. of vitamin A administered to all supplement-fed cows daily starting September 9th to November 18th. Supplement group I consisting of three cows continued at this level until end of experiment and dose to supplement group II consisting of three cows was increased to 1,400,000 I.U. of vitamin A on November 18th.

<sup>4</sup> Four experiments only in control group.

TABLE 3

*The fat content of milk in control cows receiving a basal ration throughout and in cows receiving a vitamin A supplement after a previous basal period.*

DIET	COW NO.	BUTTERFAT IN PER CENT							
		BASAL PERIOD					EXPERIMENTAL PERIOD		
		8-5	8-12	8-19	8-26	Avg.	9-16	9-23	11-4
Control group	2	4.8	5.0	5.0	5.1	5.0	5.3	5.4	5.9
	4	5.0	4.9	5.0	5.3	5.0	5.3	5.6	6.0
	5	4.7	4.6	4.8	5.0	4.8	4.4	5.1	5.4
	9	4.3	4.3	4.4	4.7	4.4	4.6	4.4	5.3
	11	3.5	3.7	3.7	3.7	3.6	3.9	4.1	4.1
	12	4.1	4.2	4.1	4.2	4.2	4.2	4.6	4.9
Average		4.4	4.4	4.5	4.7	4.5	4.6	4.9	5.5
Supplement group I	1	4.2	4.0	4.1	4.2	4.1	4.6	4.6	5.1
	6	3.9	4.1	4.0	4.3	4.1	4.4	4.8	4.7
	8	3.8	3.9	4.3	4.0	4.0	4.2	4.4	4.6
Supplement group II	3	4.3	4.4	4.4	4.5	4.4	4.5	4.6	5.2
	7	4.0	4.0	4.0	4.2	4.0	4.4	4.8	4.7
	10	4.2	4.2	4.4	4.7	4.4	4.5	4.6	4.9
Average		4.1	4.1	4.2	4.3	4.2	4.4	4.6	5.4

TABLE 4

*The beta carotene and vitamin A in the butter of Guernsey cows during a basal period followed by a period when this diet was supplemented with vitamin A.*

COW NO.	CAROTENE IN GAMMA PER GRAM <sup>1</sup>				VITAMIN A IN I.U. <sup>2</sup> PER GRAM			TOTAL VITAMIN A PER GRAM					
	Period 1 (10 cc.)		Period 2 (20 cc.)		Basal period 11-11 1940	Period 1 11-11 1940	Period 2 1-20 1941	Basal period 11-11 1940	Period 1 11-25 1940	Period 2 1-20 1941	Basal period 11-11 1940	Period 1 11-25 1940	Period 2 1-20 1941
	11-25 1940	12-16 1940	1-20 1941	11-11 1940									
13	14.4	10.0	8.3	5.8	24	33	..	33	36	42	..	38	
14	17.4	12.9	8.0	6.4	34	28	30	22	49	40	37	27	
15	14.3	10.7	7.4	5.2	13	29	32	37	25	38	39	41	
16	14.8	12.6	6.1	6.4	29	40	34	35	42	52	39	40	
17	17.7	9.8	9.1	3.1	30	34	26	29	45	42	34	32	
18	13.4	14.6	9.6	5.8	34	39	..	40	45	51	..	45	
Avg.	15.3	11.8	8.1	5.4	27	34	30	33	40	44	37	37	
Con-	14.4	....	13.2	...	24	..	44	..	36	..	55	..	
Avg.													

<sup>1</sup> Based on correction factor of 0.75.

<sup>2</sup> International Units.

indicated that the differences obtained by the physical methods were approximately correct. The bioassay for butter 5 (1-12-41) was found to be 21.2 I.U. per gram compared with a value of 31 obtained by chemical examination while the value for butter 10 (1-12-41) exceeded 155 I.U. per gram compared with 172 found on the photometer.

The increases which were noted were consistent in each case where 30 cc. of the shark liver oil or more was fed. It is surprising that it is possible to raise the level of vitamin A in the milk of cows receiving a diet of fresh-cut alfalfa designed to give the maximum levels of vitamin A in the butterfat by natural means. The vitamin A contents of butter of all the cows during the basal period and of the control cows during the experimental period which averaged from 33 to 55 I.U. per gram are in the maximum range reported for butter produced when the cows were on green pasturage.

The carotene is calculated on the basis of 1.2  $\mu$ g. being equivalent to an International Unit which would account for a 50% utilization. This seems justified on the basis of numerous reports of the comparative effectiveness of carotene and vitamin A on various species. Approximately four times as much carotene as vitamin A was required to supply an A deficiency of rats as judged by vaginal smear method (Goss and Guilbert, '39) while three times as much carotene was necessary to prevent night blindness in cattle (Guilbert, Miller and Hughes, '37). On man the effectiveness has been considered to be represented by a ratio of 1.6 to 1.8 (Booher et al., '39) and 2.0 (Wagner, '40).

Only approximately 3% of the ingested vitamin A is excreted in the milk. In view of the higher levels of vitamin A noted in the later samples of cow 10, it is possible that the efficiency of the excretion may be higher when the tissues become thoroughly saturated with this vitamin. Baumann, Steenbock, Beeson and Rupel ('34) have reported an efficiency of only 1.3% for carotene when fed at high levels.

Although a seasonal decrease in milk carotene was noted, the decrease was greater in the vitamin A-fed cows than the

controls. The decrease resulted also after the administration of the 10 cc. dosage where no increased vitamin A excretion was noted. Whether the transformation of carotene to vitamin A was accelerated in the presence of high concentrations of vitamin A or whether the lower output of this pigment resulted from a decreased intake of the carotene-containing feeds is now being investigated.

No toxic symptoms were noted by the continued ingestion of the shark liver oil for over 3 months at a level of 30 cc. daily to three cows; moreover, three cows received this level for 2 months and 60 cc. for 1 month without ill effects while one cow continued on the higher level for 3 months in apparently good health. The best indication of their nutritional condition is to be obtained from the level of their milk production which averaged approximately 10% higher than that of the controls while the butterfat excretion was increased to a somewhat greater extent (Deuel, '41). There is no evidence of the decline in butterfat in the milk similar to that caused by cod liver oil at considerably higher levels. The milk had no taste of the fish liver oil.

#### SUMMARY

The vitamin A content of the butterfat obtained from cows on a diet high in fresh alfalfa was considerably increased by the administration of shark liver oil in daily doses of approximately 700,000 I.U. although lower amounts were ineffective.

The vitamin A in butterfat averaged 113 I.U. after administration of the vitamin A supplement at a level of 1,400,000 I.U. daily. In one cow, the level reached 170 I.U. per gram which value was also noted a month later. The increased amounts of vitamin A in the butters persisted without diminution over a 5-month period during which the experiments were continued.

There is a marked decrease in carotene which occurs even when doses of shark liver oil, too small to cause an increase in the level of vitamin A in milk, are fed.

No toxic-symptoms were noted and the cows remained in good nutritional condition as reflected by the increased production of milk and butterfat.

The present experiments emphasize the lack of correlation between color of the milk and its vitamin A content.

#### LITERATURE CITED

- BAUMANN, C. A., AND H. STEENBOCK 1933 Fat-soluble vitamins. XXXVI. The carotene and vitamin A content of butter. *J. Biol. Chem.*, vol. 101, p. 547.
- BAUMANN, C. A., H. STEENBOCK, W. M. BEESON AND I. W. RUPEL 1934 Fat-soluble vitamins. XXXIX. The influence of breed and diet of cows on the carotene and vitamin A content of butter. *J. Biol. Chem.*, vol. 105, p. 167.
- BOOHER, L. E., E. C. CALLISON AND E. M. HEWSTON 1939 An experimental determination of the minimum vitamin A requirements of normal adults. *J. Nutrition*, vol. 17, p. 317.
- DEUEL, H. J., JR. 1941 *J. Dairy Science*. (In press.)
- GOLDING, J., K. M. SOAMES AND S. S. ZILVA 1926 The influence of the cow's diet on the fat-soluble vitamins of winter milk. *Biochem. J.*, vol. 20, p. 306.
- GOLDING, J. 1928 Some of the effects produced on the richness of cow milk by feeding cod liver oil. *Proc. 8th World's Congress*, p. 44.
- GOLDING, J., AND S. S. ZILVA 1928 The influence of the cow's diet on the fat-soluble vitamins of winter milk. II. *Biochem. J.*, vol. 22, p. 173.
- GOSS, H., AND H. R. GUILBERT 1939 The minimum vitamin A and carotene requirement of the rat. *J. Nutrition*, vol. 18, p. 169.
- GUILBERT, H. R., R. F. MILLER AND E. H. HUGHES 1937 The minimum vitamin A and carotene requirement of cattle, sheep, and swine. *J. Nutrition*, vol. 13, p. 543.
- HART, E. B., F. B. HADLEY AND G. C. HUMPHREY 1932 Relation of nutrition to contagious cattle abortion. *Wisconsin Agric. Exp. Sta.*, Research Bull. 112, pp. 1-45.
- HILDITCH, T. P., AND H. M. THOMPSON 1936 The effect of certain ingested fatty oils upon the composition of milk fats. *Biochem. J.*, vol. 30, p. 667.
- KLINE, O. L., M. O. SCHULTZE AND E. B. HART 1932 Carotene and xanthophyll as sources of vitamin A for the growing chick. *J. Biol. Chem.*, vol. 97, p. 83.
- KOEHN, C. J., AND W. C. SHERMAN 1940 The determination of vitamin A and carotene with the photoelectric colorimeter. *J. Biol. Chem.*, vol. 132, p. 527.
- MCCAY, C. M., AND L. A. MAYNARD 1935 The effect of ingested cod liver oil, shark liver oil, and salmon oil upon the composition of the blood and milk of lactating cows. *J. Biol. Chem.*, vol. 109, p. 29.

- McCAY, C. M., H. PAUL AND L. A. MAYNARD 1938 The influence of hydrogenation and of yeast in counteracting cod liver oil injury in herbivora, and the influence of salmon oils on milk fat secretion. *J. Nutrition*, vol. 15, p. 367.
- PETERSEN, W. E. 1932 Effect of cod liver oil in the ration on the quantity and quality of cow milk. *J. Dairy Sc.*, vol. 15, p. 283.
- WAGNER, K. H. 1940 Die experimentelle Avitaminose A beim Menschen. *Zeitschr. f. physiol. Chem.*, vol. 264, p. 153; through *Chem. Abstr.*, vol. 34, p. 5125.



# THE EFFECT OF CERTAIN ORGANIC COMPOUNDS AND OTHER DIETARY SUPPLEMENTS ON PEROSIS<sup>1</sup>

THOMAS H. JUKES

*Division of Poultry Husbandry, University of California, Davis*

TWO FIGURES

(Received for publication June 3, 1941)

Previous experiments (Jukes, '40 a, b, '41 a, b) have shown that choline is essential for the prevention of perosis in turkeys and chicks. Hogan and co-workers ('41) and Hegsted et al. ('41) have also reported evidence of the anti-perotic effect of choline on chicks. The present communication describes further experiments dealing with the relation of choline and other dietary supplements to perosis.

## EXPERIMENTAL

Single-comb White Leghorn chicks were placed in electrically-heated battery brooders at hatching and were fed the experimental diets immediately. From ten to twelve chicks were used in each group. The principal ingredients in the basal diets were crude glucose;<sup>2</sup> washed casein; a low choline strain of brewers' dried yeast;<sup>3</sup> powdered gelatin; gum arabic, U. S. P. powder; salt mixture (bone ash, 38 parts; CaCO<sub>3</sub>, 20; iodized salt, 22; MgSO<sub>4</sub>, 3.9; KH<sub>2</sub>PO<sub>4</sub>, 7.5; ferric citrate, 3.5; MnSO<sub>4</sub>, 2; copper carbonate, 0.1; ZnO, 0.1); crude soybean oil; and fish oil blend, 3000-A, 400-D.<sup>4</sup> A test period of 28 days

<sup>1</sup> Presented at the annual meeting, American Institute of Nutrition, Chicago, April, 1941.

<sup>2</sup> "Cerelose."

<sup>3</sup> Anheuser-Busch, Strain G.

<sup>4</sup> "Sardilene 400."

was usually employed. Examination for perosis was made as previously described (Jukes, '40 a, b).

Investigations by Stokstad, Almquist and co-workers ('39, '40, '41) have led to the identification of a growth factor for chicks termed the "rice factor." The rice factor was shown by these workers to consist of two components: a protein component supplying glycine and arginine and replaceable by creatine, and a carbohydrate component which could be supplied by adding gum arabic, sodium alginate, glucuronic acid, gluconic acid, galactonic lactone, arabinose or xylose to the

TABLE 1

*Effects of choline and the two components of the rice factor upon growth and perosis in chicks on a basal diet deficient in these three nutritional essentials.*

Basal diet: Glucose, 53; casein, 18; yeast, 6; salt mixture, 5; soybean oil, 5; fish oil blend, 0.03.

ADDITION TO 87 GM. BASAL DIET	INCIDENCE OF PEROSIS AT		GAIN IN 28 DAYS
	24 days	28 days	
8 gm. casein + 5 gm. glucose	0	0	71
8 gm. casein + 5 gm. gum arabic	0	0	80
8 gm. gelatin + 5 gm. glucose	57	100	66
8 gm. gelatin + 5 gm. gum arabic	62	62	90
8 gm. casein + 5 gm. glucose + 0.1 gm. choline	0	0	89
8 gm. casein + 5 gm. gum arabic + 0.1 gm. choline	0	0	75
8 gm. gelatin + 5 gm. glucose + 0.1 gm. choline	0	0	135
8 gm. gelatin + 5 gm. gum arabic + 0.1 gm. choline	0	0	162

diet. It was previously noted that turkeys (Jukes, '40 a, b) but not chicks (Jukes, '41 a) developed perosis on a diet deficient in the rice factor, and an experiment was devised to investigate the relation of choline and the two respective components of the rice factor supplied by gelatin and gum arabic to growth and perosis. The results of this experiment are shown in table 1. Perosis was not produced and the growth-promoting effect of choline was not exerted or was greatly diminished when gelatin was absent from the diet. If gelatin was added and choline omitted, the growth-promoting action of gelatin was absent. However, gum arabic was able to promote growth even in the absence of choline. Gum arabic was found to be

necessary for maximum growth, confirming the findings of Almquist, Stokstad and co-workers.

In experiment 2, gum arabic was included in the diet, and the effect of creatine as a source of the protein component of the rice factor was studied. The results are shown in table 2. Creatine, like gelatin, provoked perosis in the absence of choline. Figure 1 illustrates the tibiae of representative birds in this experiment.

In succeeding experiments the rice factor was included in the diet, which had the following composition: Glucose,<sup>5</sup> 53; washed casein, 18; gelatin, 8; yeast,<sup>6</sup> 6; gum arabic, 5; salt

TABLE 2

*Effects of choline and creatine on growth and perosis*

Basal diet: Glucose, 53; casein, 26; yeast, 6; salt mixture, 5; gum arabic, 5; soybean oil, 5; fish oil blend, 0.3.

ADDITION TO 100 GM. BASAL DIET	INCIDENCE OF PEROSIS AT		GAIN IN 28 DAYS gm.
	21 days	28 days	
None	% 14	% 14	83
0.1 gm. choline	0	0	82
1.0 gm. creatine	50	75	106
0.1 gm. choline + 1.0 gm. creatine	0	0	180

mixture, 5; soybean oil, 5; fish oil blend,<sup>7</sup> 0.3 (diet 115). This diet was used in subsequent experiments on choline deficiency in chicks. On this diet it was found that chicks usually averaged between 100 and 120 gm. in weight at 28 days with an incidence of perosis of 80% to 100%. Usually the growth rate was approximately doubled and perosis was prevented completely by adding 0.1% of choline to the diet. However, this level of choline is marginal for the prevention of perosis under such conditions, and occasionally a mild case appeared. The growth-promoting effect of choline was evident to a significant degree when choline was added at levels as low as 0.01% which had no discernible effect on perosis.

<sup>5</sup> See footnote 2.

<sup>6</sup> See footnote 3.

<sup>7</sup> See footnote 4.

The effect of a secondary deficiency in preventing the appearance of perosis was also noted under a different set of conditions. In this case, gelatin was included in the basal diet but casein was omitted, and a sample of meat scrap was used as the source of protein. Simultaneously, part of the

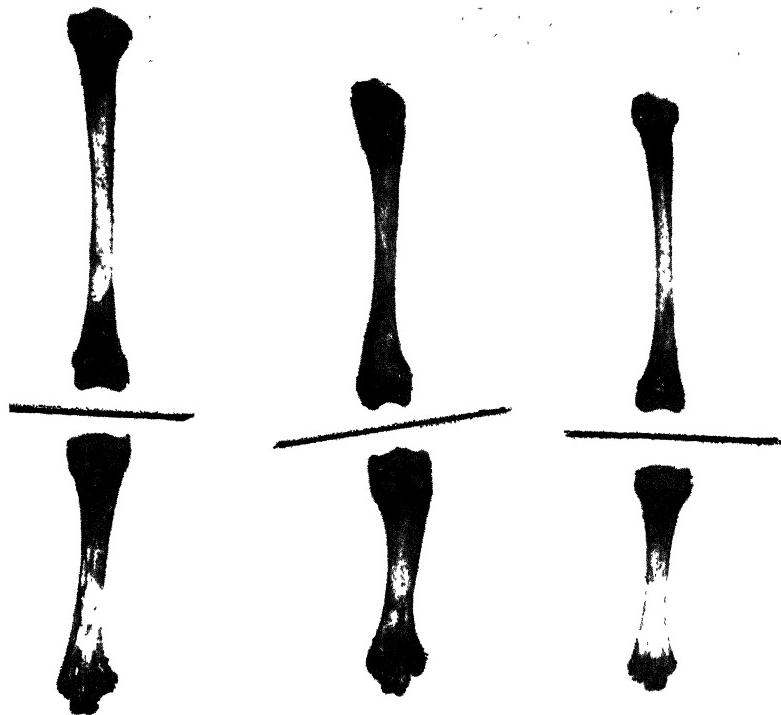


Fig. 1 Tibiae and metatarsi of chicks in experiment 2 at 28 days of age on (a) basal diet + 1% creatine + 0.1% choline, (b) basal diet + 1% creatine, (c) basal diet. The lines have been added to indicate the direction of the transverse plane of the hock joints.

minerals were omitted because of the high mineral content of the meat scrap. Growth was very slow and was not increased by the addition of choline. Perosis did not appear. However, when casein was added and choline omitted, perosis was produced. The experimental data are summarized in table 3.

Evidently casein "provoked" perosis in a manner similar to gelatin, although gelatin was present in the basal diet.

*Experiments with manganese deficiency.* If manganese was omitted from the salt mixture, diet 115 contained only 2.3 p.p.m. of manganese, which is not sufficient to protect against perosis (Gallup and Norris, '39). An experiment was made with this modification of the basal diet and the results are

TABLE 3

*Aggravating effect on perosis of casein when added to a diet in which meat scrap was the principal protein source.*

SERIES NO.	DIET NO.	CHANGES IN DIET 115	INCIDENCE OF PEROSIS AT 28 DAYS	GAIN IN gm. IN 28 DAYS
1	1	None	71	48
1	2	22 gm. meat scrap + 0.5 gm. NaCl + 0.05 gm. MnSO <sub>4</sub> replacing 18 gm. casein + 5 gm. salt mixture	0	37
1	3	Diet no. 2 + 0.1% choline	0	38
1	4	Diet no. 1 + 0.1% choline	0	152
2	1	None	89	76
2	5	Diet no. 2 + 8% casein	56	118
2	4	Diet no. 1 + 0.1% choline	0	150

illustrated in figure 2. Perosis developed and growth was slow in the absence of either manganese or choline. The appearance of the perosis in the manganese-deficient birds was similar to that in the choline-deficient birds. This confirms the observations of Hogan, Richardson, Patrick and Kempster ('41) who found that manganese and an organic factor were both necessary for the prevention of perosis.

It was suggested (Jukes, '41 a) that the tension exerted on the bones by the muscles may play a part in causing the distortion of the bones which characterizes perosis and if the tension is reduced by muscular dystrophy as in creatine deficiency, the tendency toward perosis may be lessened. This suggestion was made as a result of the observation that perosis did not develop on a diet which was deficient both in creatine or one or more of its precursors and in choline. As

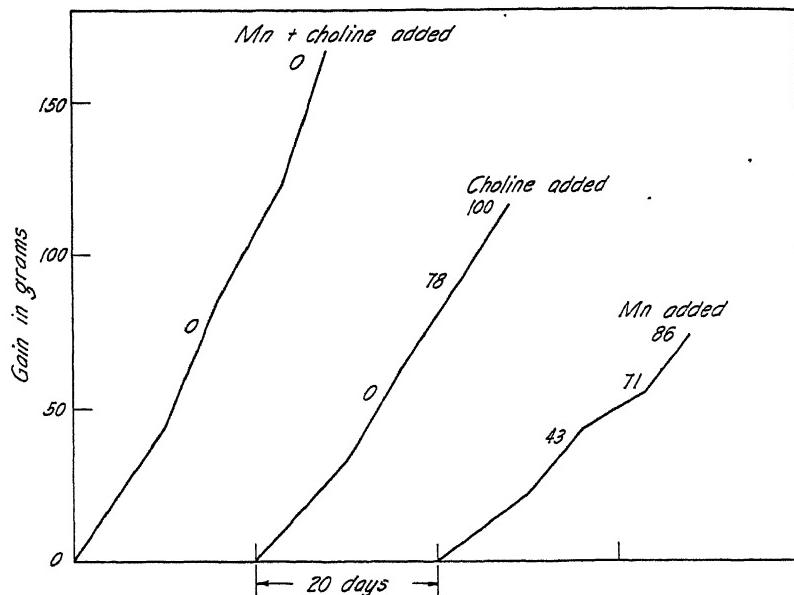


Fig. 2 Effects on chicks of additions of manganese and choline, separately and in combination, to a diet deficient in both these substances. The figures above the curves indicate the per cent incidence of perosis.

a further test of the suggestion, an experiment was made with a diet from which manganese and gelatin were omitted. The results are shown in table 4. Creatine deficiency reduced somewhat the severity of the perosis caused by a lack of manganese but the effect was not so clear-cut as that obtained

TABLE 4

*Effects upon growth and perosis in chicks produced by adding choline but omitting manganese and gelatin from diet 115.*

Basal diet: Glucose, 53; casein, 18; yeast, 6; gum arabic, 5; salt mixture (Mn omitted), 5; soybean oil, 5; fish oil blend, 0.3; choline, 0.1.

ADDITION TO 92 GM. BASAL DIET	INCIDENCE OF PEROSIS AT			GAIN IN 28 DAYS gm.
	15 days	20 days	28 days	
8 gm. casein	14	43	43	89
8 gm. gelatin	33	78	100	116
8 gm. casein + 0.1 gm. MnSO <sub>4</sub>	0	0	0	111
8 gm. gelatin + 0.1 gm. MnSO <sub>4</sub>	0	0	0	166

in the case of perosis caused by a lack of choline (Jukes, '41 a, also vide supra).

*Effect of methionine.* Methionine was shown by Tucker and Eckstein ('37) to have a lipotropic action similar to that of choline (Best and Huntsman, '32). Griffith and Mulford ('41) found that methionine could replace choline in the prevention of hemorrhagic kidneys in young rats, 1 part of choline being replaceable by 3 to 4 parts of methionine. A direct explanation of these phenomena is probably furnished by the observation that methionine may act as a precursor of choline in vivo in the case of rats (du Vigneaud, Chandler, Cohn and Brown, '40). However, methionine at a level of 0.2% was found to be ineffective as a substitute for choline in the

TABLE 5

*Effects of additions of methionine, methionine plus aminoethanol, and choline to diet 115.*

ADDITION TO 100 GM. DIET 115	INCIDENCE OF PEROSIS AT		GAIN IN 24 DAYS
	15 days	24 days	
None	%	%	gm.
0.5 gm. methionine	80	89	64
0.5 gm. methionine + 0.3 gm. aminoethanol	67	75	62
0.1 gm. choline	83	89	67
	0	0	107

prevention of perosis in turkeys (Jukes, '41 b). In view of the results of Griffith and Mulford, it seemed desirable to perform another experiment with methionine and to feed it to chicks at a higher level than was used previously. Aminoethanol was also added to the diet of one group with the thought that methionine might furnish the methyl groups (du Vigneaud, Chandler, Cohn and Brown, '40) and aminoethanol the remaining moiety of the choline molecule. The results are shown in table 5. Methionine was completely ineffective as a precursor of choline, which is not surprising in view of the fact that acute choline deficiency in chicks is produced by diet 115 which contains 18% of casein. Casein contains about 3% of methionine (Baernstein, '36).

*Extraction of the anti-perotic factor from natural foods.* Choline exists in nature largely in the form of phospholipins such as lecithin which is to some extent in loose combination with proteins. In such combination, lecithin is not extractable with fat solvents until the lecithoproteins have been subjected to vigorous treatment, such as boiling with alcohol. The action of boiling alcohol on soybean meal was investigated as follows: 1 kilo of commercial soybean meal was boiled for 1 hour with 4 liters of 95% ethanol. The mixture was filtered, and the residue was washed with hot ethanol. The entire process was repeated, and the residue was washed with hot ethanol. The residual soybean meal was dried at room temperature. The

TABLE 6

*Effect of extraction with boiling alcohol upon the anti-perotic activity of soybean meal.*

SUPPLEMENT TO DIET 115	INCIDENCE OF PEROSIS AT		GAIN IN 28 DAYS
	21 days	28 days	
None	%	%	gm.
Residue from 30% soybean meal <sup>1</sup>	75	100	74
30% original soybean meal <sup>1</sup>	70	90	141
30% original soybean meal <sup>1</sup>	0	0	202
0.1% choline	0	0	142

<sup>1</sup> Replacing 12 parts of casein and 18 parts of cerelose in diet 115.

combined alcoholic extracts were evaporated to small volume under reduced pressure. The residue was compared with the original soybean meal in a feeding experiment which is summarized in table 6. Treatment with alcohol evidently removed the anti-perotic factor from soybean meal, but the residue still supplied a growth-promoting fraction which was absent from the basal diet. The alcohol-soluble material was fed in a separate experiment and was found to promote growth and protect against perosis almost completely when fed at a level corresponding to 40% of soybean meal. An alcoholic extract was similarly prepared from meat scrap and was found to be growth-promoting and partially protective against perosis. When fed at a level corresponding to 30% meat scrap,

the effect was similar to that obtained from about 0.07% choline. These results do not show that the anti-perotic factor in soybean meal and meat scrap is identical with choline, but the experiments indicate that the factor has certain properties corresponding to those of choline.

#### DISCUSSION

It was observed (Jukes, '39) that manganese under certain dietary conditions accelerated the appearance of perosis in turkey poult's. Later it was found that the basal diet used was deficient in choline, and thus it became evident that high levels of manganese had no "sparing action" on choline.

The complete and surprising inability of methionine to replace choline in the case of the chick serves to contrast this species with the rat. The anti-perotic activity of foods, hence, cannot be related to their methionine content. This statement is further supported by the observation that the anti-perotic activity of soybean meal and meat scrap was removed by boiling alcohol which would not extract methionine from the proteins of these materials.

In this investigation, as previously (Jukes, '41 a), the feathers of birds on diets deficient in creatine or its precursors were "frayed" (Warren, '38) in appearance due at least partly to imperfect development of the barbules and sparseness of the barbs. The same appearance was noted to a marked degree in birds on diets 2 and 3 in table 3, which contained no casein.

Perosis has also been observed (McElroy and Jukes, '40) as a symptom accompanying the egg white syndrome in chicks. The basal diet contained manganese, and in one experiment 0.5% of choline was added to the basal diet without any noticeable effect in preventing perosis. The perotic symptoms were characterized by a marked distortion of the shafts of the tarsi, recalling the symptoms described by Buckner et al. ('32), rather than by the looseness and dislocation of the tibio-tarsal joints noted in the cases of deficiencies of manganese or choline.

Several nutritional conditions are now known to affect perosis. Perosis is aggravated by dietary creatine and its precursors in gelatin and may also be aggravated under certain conditions by casein. Perosis may be produced or aggravated by the addition of raw egg white to the diet, and by the addition of excessive quantities of mineral supplements such as bone meal (Card, '29), magnesium carbonate (Buckner et al., '32), and ferric citrate (Wilgus and Patton, '39). Perosis is prevented by manganese (Wilgus, Norris and Heuser, '36), by choline either free or in the form of lecithin (Jukes, '41 b), by certain choline analogues (Jukes, '40 b; Jukes and Welch, unpublished), and possibly by certain unidentified factors.

#### SUMMARY

1. A diet is described for the production of acute choline deficiency in chicks. The deficiency is characterized by slow growth and perosis.
2. Perosis did not develop if creatine or gelatin was omitted from the basal diet, nor did perosis develop if the casein in the basal diet was substituted by meat scrap, but if casein and meat scrap were both included perosis developed.
3. Manganese and choline were both found to be necessary for growth and for the prevention of perosis in chicks.
4. Methionine at a level of 0.5% was found to be without effect on choline deficiency in chicks, although 0.1% choline was sufficient to prevent perosis and promote growth when added to the basal diet. This observation contrasts chicks with rats, which are protected from choline deficiency by methionine.
5. The anti-perotic activity of certain natural foods was measured by feeding them in combination with the basal choline-deficient diet. The activity was removed from soybean meal and meat scrap by extraction with boiling alcohol.
6. The nutritional factors affecting perosis are enumerated.

The assistance of Robert E. Ranney and Russell A. Donogh is gratefully acknowledged. Thanks are given to the Research Corporation of New York for a grant in partial support of

these studies. Acknowledgment is gratefully made of gifts of casein by the Golden State Company, San Francisco, of yeast by Anheuser-Busch, Inc., St. Louis, and of gelatin by the Gelatin Products Company of Detroit.

#### LITERATURE CITED

- ALMQUIST, H. J., AND E. MECCHI 1940 Identification of the rice factor. The essential nature of the glycine component. *J. Biol. Chem.*, vol. 135, p. 355.
- ALMQUIST, H. J., E. L. R. STOKSTAD, E. MECCHI AND P. D. V. MANNING 1940 Identification of the rice factor. *J. Biol. Chem.*, vol. 134, p. 213.
- ALMQUIST, H. J., E. MECCHI, E. L. R. STOKSTAD AND P. D. V. MANNING 1940 Identification of the rice factor. The carbohydrate component. *J. Biol. Chem.*, vol. 134, p. 465.
- BAERNSTEIN, H. D. 1936 A new method for the determination of methionine in proteins. *J. Biol. Chem.*, vol. 115, p. 25.
- BEST, C. H., AND M. E. HUNTSMAN 1932 The effects of the components of lecithin upon the deposition of fat in the liver. *J. Physiol.*, vol. 75, p. 405.
- BUCKNER, G. D., J. H. MARTIN AND W. M. INSKO 1932 The effect of magnesium carbonate when added to diets of growing chicks. *Poultry Sci.*, vol. 11, p. 58.
- CARD, L. E. 1929 Mistake to feed extra minerals to chicks. *Ill. Agr. Exp. Sta. Annual Report*, p. 113.
- DU VIGNEAUD, V., J. P. CHANDLER, M. COHN AND G. B. BROWN 1940 The transfer of the methyl group from methionine to choline and creatine. *J. Biol. Chem.*, vol. 134, p. 787.
- GALLUP, W. D., AND L. C. NORRIS 1939 The amount of manganese required to prevent perosis in the chick. *Poultry Sci.*, vol. 18, pp. 76-82.
- GRIFFITH, W. H., AND D. J. MULFORD 1941 Choline metabolism. VI. Hemorrhagic degeneration. *J. Am. Chem. Soc.*, vol. 63, p. 929.
- HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. *J. Biol. Chem.*, vol. 138, p. 459.
- HOGAN, A. G., L. R. RICHARDSON, H. PATRICK AND H. L. KEMPSTER 1941 Perosis due to a vitamin deficiency. *J. Nutrition*, vol. 21, p. 327.
- JKUES, T. H. 1939 Ineffectiveness of manganese in preventing slipped tendon in turkey poult. *Poultry Sci.*, vol. 18, p. 405.
- 1940 a Prevention of perosis by choline. *J. Biol. Chem.*, vol. 134, p. 789.
- 1940 b Effect of choline and other supplements on perosis. *J. Nutrition*, vol. 20, p. 445.
- 1941 a Effects of choline, gelatin and creatine on perosis in chicks. *Proc. Soc. Exp. Biol. and Med.*, vol. 46, p. 155.
- 1941 b Studies of perosis in turkeys. I. Experiments related to choline. *Poultry Sci.*, vol. 20, p. 251.

- MCELROY, L. W., AND T. H. JUKES 1940 Formation of the anti-egg-white-injury factor (biotin) in the rumen of the cow. *Proc. Soc. Exp. Biol. and Med.*, vol. 45, p. 296.
- STOKSTAD, E. L. R., H. J. ALMQVIST, E. MECCINI, P. D. V. MANNING AND R. E. ROGERS 1941 The carbohydrate component of the rice factor. *J. Biol. Chem.*, vol. 137, p. 373.
- STOKSTAD, E. L. R., AND P. D. V. MANNING 1939 Studies on a growth factor present in polished rice and required by chicks on a simplified diet. *Poultry Sci.*, vol. 18, p. 413.
- TUCKER, H. F., AND H. C. ECKSTEIN 1937 The effect of supplementary methionine and cystine on the production of fatty livers by diet. *J. Biol. Chem.*, vol. 121, p. 479.
- WARREN, D. C. 1939 A heritable variation of feather structure in the fowl. *J. Heredity*, vol. 29, p. 91.
- WILGUS, H. S., JR., L. C. NORRIS AND G. F. HEUSER 1936 The role of certain inorganic elements in the cause and prevention of perosis. *Science*, vol. 84, p. 252.
- WILGUS, H. S., JR., AND A. R. PATTON 1939 Factors affecting manganese utilization in the chick. *J. Nutrition*, vol. 18, p. 35.

# INFLUENCE OF PROTEIN INTAKE UPON GROWTH, REPRODUCTION, AND LONGEVITY STUDIED AT DIFFERENT CALCIUM LEVELS<sup>1</sup>

HSUEH-CHUNG KAO, R. T. CONNER AND H. C. SHERMAN

*Department of Chemistry, Columbia University, New York*

(Received for publication May 3, 1941)

The present paper completes a series of three reports upon our investigation of the effects of simultaneous increases of the protein and calcium contents of a diet already adequate, but suboptimal in calcium, and perhaps also in its protein and riboflavin contents and vitamin A value. Increasing the original 14.4% of protein to 18.8 or to 25% by addition of casein increased the rate of growth and almost correspondingly the amount of calcium in the body at a given age, but the percentage of body calcium was not thereby increased (Conner and Sherman, '36; Conner, Kao and Sherman, '41).

The effects of these increments of protein intake upon the nutritional well-being of animals throughout their life cycles have now been studied at two liberal levels of calcium intake: 0.64 and 0.80% of calcium in the air-dry food mixture. The basal dietary of this investigation is more or less closely analogous to widely prevalent conditions of human food supply. The outcome of these experiments should, therefore, have a significant bearing upon the general problem as to what degrees of simultaneous enrichment of the dietary in calcium and protein are most conducive to permanent nutritional well-being.

Our problem is thus quite different from that of Slonaker ('38, '39), who studied the effects of increasing the protein

<sup>1</sup> The assistance of a grant from The Rockefeller Foundation is gratefully acknowledged.

TABLE I  
Records of rats on diets of different protein and calcium contents.

Protein in diet	Calcium in diet	DIET 170		DIET 171		DIET 673		DIET 172		DIET 675	
		18.8%		18.8%		25.0%		18.8%		25.0%	
		No. of cases									
<b>Weight at 30 days</b>											
Males, gm.	35	52 ± 0.9	52	55 ± 0.5	63	59 ± 0.5	45	51 ± 0.7	51	56 ± 0.6	
Females,,	40	50 ± 0.7	70	51 ± 0.5	68	55 ± 0.5	58	48 ± 0.6	59	54 ± 0.6	
<b>Weight at 60 days</b>											
Males, gm.	30	137 ± 2	47	153 ± 2	51	169 ± 2	41	150 ± 2	39	157 ± 2	
Females,,	34	112 ± 1	65	118 ± 1	56	131 ± 1	53	121 ± 1	47	128 ± 2	
<b>Weight at 90 days</b>											
Males, gm.	24	200 ± 3	42	226 ± 2	39	239 ± 3	36	230 ± 2	29	224 ± 4	
Females,,	28	155 ± 3	60	168 ± 3	44	175 ± 2	47	174 ± 2	31	170 ± 2	
<b>Weight at 180 days</b>											
Males, gm.	15	306 ± 4	33	323 ± 3	27	312 ± 4	27	324 ± 4	15	291 ± 6	
Females,,	16	207 ± 2	46	214 ± 2	32	214 ± 3	38	213 ± 2	19	203 ± 3	
<b>Weight at 365 days</b>											
Males, gm.	7	349 ± 5	28	355 ± 5	13	367 ± 6	22	364 ± 4	4	361 ± 21	
Females,,	9	252 ± 3	39	253 ± 2	20	254 ± 4	33	254 ± 2	4	254 ± 5	
<b>Age of females at birth of first young, days</b>											
Duration of reproductive life, days	11	124 ± 5.4	40	102 ± 1.4	18	101 ± 1.9	32	104 ± 2.1	6	100 ± 3.7	
Young born per female	11	267 ± 30	41	319 ± 9	21	328 ± 24	33	378 ± 13	6	302 ± 37	
Young reared per female	11	36 ± 4.3	41	43 ± 2.9	21	44 ± 3.3	33	48 ± 1.7	6	45 ± 6.2	
Average weight of young at 28 days, gm.	25.5	44.5 ± 0.2	1185	47.5 ± 0.1	578	48.8 ± 0.1	10.51	46.9 ± 0.1	20.5	48.4 ± 0.2	
<b>Length of life</b>											
Males, days	8	674 ± 39	28	714 ± 21	14	684 ± 32	22	720 ± 16	4	699 ± 54	
Females,,	11	709 ± 59	40	848 ± 19	21	831 ± 22	33	883 ± 15	6	731 ± 80	

content of the diet by addition of lean meat only; and our experiments differ essentially from those of Steenbock, Kent and Gross ('18) in that we have employed not only a different basal diet but also a series of criteria of nutritional well-being which extend to all parts of the normal life cycle. The conditions here are also very different from those of McCay, Maynard, Sperling and Osgood ('41) whose feedings of different levels of protein were begun when the experimental animals were middle-aged.

#### EXPERIMENTAL

Strictly parallel lots of laboratory-bred rats of the uniform initial age of 4 weeks (three females and two males in each lot) were fed the five diets whose effects are here compared. The protein and calcium contents of each diet are shown in table 1 and the full description may be found, if desired, in the paper of Conner and Sherman ('36, pp. 697 and 704). The original matched lots of experimental animals were continued on their respective diets from the age of 4 weeks (here considered the end of infancy in the rat) until the completion of their natural lives. Many of their offspring were continued on the same family dietaries throughout their entire lives, and in the case of all the diets here discussed except diet 675, typical members of the third generation were also continued on the family dietary. None of the dietaries here studied resulted in any marked change from one generation to another; hence, in our table 1 we give the mean results of the records of all the rats directly compared on the respective diets without distinction between the individuals of the first, second, and third generations.

#### DISCUSSION

The data shown in table 1, being the results of side-by-side experiments with directly matched initial animals, are strictly comparable. With moderate allowance for experimental variation, these may also be compared with the data for closely related animals which at practically the same time were fed diets 168 and 169 containing 14.4% of protein and, respec-

tively, 0.64 and 0.80% of calcium, as described in another paper from this laboratory (Van Duyne, Lanford, Toepfer and Sherman, '41).

The rate of growth and correspondingly the size at early ages tend to increase with the level of protein fed, but the adult size is essentially the same with 18.8 or 25% of protein, and only slightly greater than with 14.4%.

Similarly the higher protein levels seem to result in very slightly earlier maturity as judged by the age of the females at birth of their first young, but the differences here, particularly between the animals on 18.8 and 25.0% protein, were not much larger than their probable errors and are therefore of doubtful significance.

The higher intakes of protein seem to result in slightly larger offspring, the difference appearing more clearly significant between the animals on diets with 14.4 and 18.8% than between those receiving 18.8 and 25% of protein in the dry food mixture. Thus at the calcium level of 0.61–0.64%, the average weights of the offspring at 28 days were, on the three protein levels,  $40.2 \pm 0.1$ ,  $47.5 \pm 0.1$ , and  $48.8 \pm 0.1$  gm., respectively. A similar relationship holds for the results of the three levels of protein feeding when the calcium content of the dietaries is 0.77–0.81%.

Thus the optimal degree of enrichment with protein seems to have been reached at a level of about 18% of protein in dietaries which also contained the favorable calcium content of about 0.6% of the dry food. These figures seem clearly to be within the range of the optimal plateau.

#### CONCLUSIONS

Within the range here studied, 14.4 to 25% of protein in the dry food mixture, increased protein content of the diet tended to increase growth up to about 60 days of age in the rat. This was found for both sexes and for both the basal diets used, containing respectively 0.61–0.64 and 0.77–0.81% of calcium in the dry matter.

At 6 months and at 1 year of age the rats which had received diets with 18.8 and 25% of protein respectively, were of essentially the same size, and slightly larger than those which received the diet with 14.4% protein.

With the higher protein intakes, maturity appeared to be reached slightly earlier as judged by the age of the females at birth of first young.

The average numbers of young born, and of young reared, per female, were essentially the same on the diets of 18.8 and 25% protein respectively. The duration of reproductive life was also essentially the same on the different protein levels here studied. The higher levels of protein resulted in slightly higher average weight of young at 28 days of age.

With each sex and with each calcium level, the average length of life was essentially the same on the different protein levels here studied.

#### LITERATURE CITED

- CONNER, R. T., H.-C. KAO AND H. C. SHERMAN 1941 Further studies on the relationship of the plane of protein intake to the rate of normal calcification during growth. *J. Biol. Chem.*, vol. 139, pp. 835-841.
- CONNER, R. T., AND H. C. SHERMAN 1936 Some aspects of protein intake in relation to growth and rate of calcification. *J. Biol. Chem.*, vol. 115, pp. 695-706.
- MCCAY, C. M., L. A. MAYNARD, G. SPERLING AND H. S. OSGOOD 1941 Nutritional requirements during the latter half of life. *J. Nutrition*, vol. 21, pp. 45-60.
- SLONAKER, J. R. 1938 The effect of different per cents of protein in the diet in successive generations. *Amer. J. Physiol.*, vol. 123, pp. 526-542.
- 1939 The effect of different percentages of protein in the diet of six generations of rats. *Stanford Univ. Publ., Univ. Series, Biological Sciences*, vol. VI, no. 4, pp. 257-321.
- STEENBOCK, H., H. E. KENT AND E. G. GROSS 1918 The dietary qualities of barley. *J. Biol. Chem.*, vol. 35, pp. 61-74.
- VAN DUYNE, F. O., C. S. LANFORD, E. W. TOEPFER AND H. C. SHERMAN 1941 Life-time experiments upon the problem of optimal calcium intake. *J. Nutrition*, vol. 21, pp. 221-224.



THE INFLUENCE OF PLANE OF NUTRITION AND  
OF ENVIRONMENTAL TEMPERATURE ON THE  
RELATIONSHIP BETWEEN BASAL META-  
BOLISM AND ENDOGENOUS NITROGEN  
METABOLISM SUBSEQUENTLY  
DETERMINED<sup>1</sup>

RAY TREICHLER AND H. H. MITCHELL

*Animal Nutrition Division, University of Illinois, Urbana*

(Received for publication June 23, 1941)

The demonstration by Terroine and Sorg-Matter ('27), amply confirmed by Smuts ('35) and by Brody, Procter and Ashworth ('34), that there is a relationship approaching constancy between the endogenous nitrogen metabolism and the basal metabolism in adult animals maintained under comparable conditions imposing no strain upon the heat regulating mechanism, is a most important scientific contribution with important practical applications. Since the endogenous loss of nitrogen is the predominant component among the factors determining the maintenance requirement of protein, the constancy of this ratio among adult animals means that the maintenance requirement of protein varies with body size in a manner similar to the basal metabolism, i.e., in proportion to body surface, not in proportion to body weight as is all too generally presumed.

The importance of this relationship justifies an extension of its study to immature animals and to animals under variable environmental conditions. This paper reports the results of experiments on rats on the effect of variable caloric intake

<sup>1</sup> The data reported in this paper were taken from a thesis presented by Ray Treichler to the Graduate School of the University of Illinois in 1939 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Animal Husbandry.

and of low environmental temperature on the basal metabolism and the endogenous nitrogen metabolism and on the ratio of the two. These variable conditions were imposed for a period of time prior to the measurements themselves, which were carried out according to the usual standardized procedures. The effect of variable age will be reported later.

The imposition of low environmental temperature prior to the basal metabolism determination has been shown to exert a stimulating effect, direct or indirect, on the basal metabolism of albino rats by Benedict and MacLeod ('29), Giaga and Gelineo ('30), Ring ('36, '39), Schwabe and Griffith ('36), Schwabe, Emery and Griffith ('38), and Collip and Billingsley ('36, '37). A stimulating effect of low temperature on the endogenous nitrogen excretion has been reported by Terroine and Sorg-Matter ('28) on mice and by Fontaine, Guillemet and Mandel ('38) on dogs. But whether the two effects are proportional so that the constancy of the ratio of endogenous nitrogen to basal heat is maintained, awaits simultaneous measurements on the same animals.

The plane of nutrition has also been found to exert an influence on the basal metabolism of the albino rat. Particularly evident has been the depressing effect of undernutrition (Gulick, '22, '24; Aszodi, '24; Horst, Mendel and Benedict, '34). With other species of animals, also, including man, undernutrition has generally been found to be associated with a subnormal metabolic rate. However, a stimulating effect of overnutrition has not been generally observed. For the growing albino rat, Hamilton ('37) has shown conclusively that there is a direct and close relationship between variations in the previous caloric intake and variations in the basal metabolism. No evidence seems to be at hand on the effect of plane of nutrition on the endogenous nitrogen metabolism.

#### PLAN OF THE EXPERIMENT

The investigation involved a study of the effect of varying two factors, (a) the plane of nutrition, and (b) the environmental temperature, upon subsequent determinations of basal

metabolism and endogenous nitrogen excretion in the urine carried out according to standard procedures.

Except for those animals subjected to subcritical temperatures, all experimental animals were maintained at an environmental temperature of approximately 28°C. Basal metabolism and endogenous nitrogen excretion were measured at, or slightly above, 28°C. In the test on the effects of low environmental temperature, the animals were maintained at approximately 4°C. for a minimum of 1 week prior to the experimental measurements. The level of food intake was maintained constant as closely as possible throughout the entire experimental period. The experimental diets are described in table 1.

TABLE I  
*Percentage composition of experimental diets.*

INGREDIENTS	DIET 293 10% PROTEIN	DIET 292 4% PROTEIN	DIET 296 PROTEIN- FREE
Dried ether-extracted whole egg	14.69	5.82	....
CellufLOUR <sup>1</sup>	4.00	4.00	4.00
Cornstarch	48.31	57.18	57.18
Sucrose	10.00	10.00	15.82
Wesson salts <sup>2</sup>	5.00	5.00	5.00
Corn oil	8.00	8.00	8.00
Butterfat	8.00	8.00	8.00
Cod liver oil	1.50	1.50	1.50
Wheat germ oil	0.50	0.50	0.50
Per cent nitrogen <sup>3</sup>	1.68	0.69	0.04
Calories per gram <sup>3</sup>	4.80	4.65	4.57

<sup>1</sup> Product of the Chicago Dietetic Supply House. Contains 37.8% crude fiber and 0.015% nitrogen.

<sup>2</sup> Wesson ('32) slightly modified to contain traces of cobalt and zinc.

<sup>3</sup> These values are averages of all the diets made up during the course of the experiment.

In the first experiment, sixteen mature male albino rats were selected. They were separated into two groups of eight each, and so fed that at the time of the basal energy determinations they approximated each other in weight and age. The one group (rats 37-44, inclusive) was fed diet 293 for mainte-

nance of weight only, while the other group (rats 45-52, inclusive) received the same diet at a higher caloric intake, which permitted a slow gain.

In the second experiment, which involved variation of both caloric intake and environmental temperature, fifteen male albino rats were used. They were separated into five trios, the rats in each trio approximating each other in age and weight. One rat in each trio was fed diet 293 at a level required for maintenance of body weight and at an environmental temperature of approximately 4°C. The second rat in each trio received the same amount of the same diet, or as close to the same amount as it was possible to get it to eat, at an environmental temperature of 28°C. The third rat in each trio was fed maintenance amounts of diet 293 at an environmental temperature of 28°C.<sup>2</sup>

In determining the endogenous level of nitrogen excretion in the urine, collections were obtained both on the 4% egg-protein diet (no. 292) and on the protein-free diet (no. 296) with quite comparable results. Ferric oxide was used in all cases to separate the fecal collections.

Basal energy determinations were made by the gravimetric method of Haldane (1892) as modified by Mitchell and Carman ('26). Those rats maintained at an environmental temperature of 4°C. were removed to an environmental temperature of 28°C. 4 hours prior to the start of the basal energy measurement. Immediately preceding and following basal energy measurements, the body temperature was taken, using a rectal clinical thermometer inserted ad rectum. Body temperature was taken over a 7-week period on those rats used in the environmental temperature and food intake study. The details of the various technical procedures used in this experiment were the same as those described by Smuts ('35).

#### EXPERIMENTAL RESULTS

The results of the first experiment are summarized in table 2. The average basal metabolic rate of the rats on the higher

<sup>2</sup>The relative humidities at the two experimental temperatures were 76% in the cold room and 32% in the warm room.

TABLE 2

*Summary of results on rats receiving different caloric intakes. Experiment I.*

	RATS 37-44	RATS 45-52	SIGNIFICANCE OF MEAN DIFFERENCE <sup>2</sup>		
	s	t	Odds		
Weight, gm.	231	252			
Food intake, cal.	37.51	45.34			
Basal heat, cal./m <sup>2</sup> /day <sup>1</sup>	707	766	47.74	2.44	62:1
Endogenous N, mg./m <sup>2</sup> /day <sup>1</sup>	1535	1472	64.37	1.98	27:1
Total creatinine N, pct. of endogenous N, pct.	8.00	7.79	0.45	0.9	4.7:1
Ratio of endogenous N to basal heat, mg./cal.	2.18	1.85	0.25	2.64	>100:1

<sup>1</sup> Body surfaces were estimated from body weight by the formula of Lee ('29):

$$S_{\text{cm}^2} = 1254 W^{.6}$$

<sup>2</sup> R. A. Fisher: Statistical Methods for Research Workers. London. 1928. 2nd ed. P. 107.

plane of nutrition was 8.3% higher than that of the rats on the lower plane of nutrition and the difference was highly significant statistically. That it was a result of the higher plane of nutrition rather than of a greater growth rate may be inferred from the experiments of Hamilton ('37), which showed that different rates of growth unassociated with different caloric intakes do not induce variations in the basal metabolic rate in rats.

The endogenous nitrogen output in the urine per square meter of body surface averaged about 4% less on the higher plane of nutrition, though the odds favoring the significance of this difference are not sufficiently great to permit a very positive conclusion. The proportion of total creatinine nitrogen to total endogenous nitrogen was obviously not affected by the plane of nutrition.

As a consequence of the effects of the plane of nutrition on the basal metabolic rate on the one hand, and on the endogenous nitrogen output on the other, the ratio of endogenous nitrogen to basal calories was considerably and significantly depressed by the higher caloric intake, the average ratios being 2.18 on the lower plane of nutrition and 1.85 on the higher.

TABLE 3  
*Summary of data obtained in studies involving different environmental temperatures and food intake levels.*

TRIO	GROUP NO. <sup>1</sup>	RAT NO.	INITIAL WEIGHT	AVERAGE DAILY CALORIC INTAKE		BASAL HEAT <sup>a</sup>	ENDOGENOUS NITROGEN	TOTAL CREATININE N PER CENT OF ENDOGENOUS N	RATIO ENDOGENOUS N TO BASAL HEAT
				Prior to basal	During collection				
1	1	53	250	78	83	876	1728	5.90	1.97
	2	54	256	68	50	871	1474	8.55	1.69
	3	55	241	39	40	736	1558	8.27	2.12
2	1	56	214	75	78	760	1946	5.88	2.56
	2	57	213	59	78	763	1356	8.46	1.78
	3	58	205	35	32	689	1386	7.88	2.01
3	1	59	260	78	83	863	1840	5.24	2.13
	2	60	266	71	54	846	1210	9.98	1.43
	3	61	263	37	32	636	1280	8.81	2.01
4	1	62	233	77	83	819	1493	6.17	1.82
	2	63	243	71	52	900	1526	5.96	1.70
	3	64	250	35	37	619	1155	9.47	1.87
5	1	65	237	79	82	809	1573	6.29	1.94
	2	66	238	64	41	821	1411	7.12	1.72
	3	67	238	35	37	712	1229	9.22	1.73
Aver.	1		239	77	82	825	1716	5.90	2.08
	2		243	67	55	840	1395	8.01	1.66
	3		239	36	36	678	1322	8.73	1.95

<sup>1</sup> 1 = 4°C., maintenance; 2 = 28°C., excess food; 3 = 28°C., maintenance.

The data obtained in the second experiment<sup>3</sup> are summarized in table 3, while the results of the statistical treatment of the permissible comparisons are presented in table 4.

The first comparison in table 4 shows that the basal metabolic rate is significantly increased by prior exposure of the rat to a low environmental temperature, the food intake being adjusted at both temperatures to maintain a constant body

<sup>a</sup> The mean body temperatures for the various groups of rats in the second experiment were as follows: 4°C. maintenance  $38.3 \pm .06^\circ\text{C}$ .; 28°C. maintenance  $38.5 \pm .06^\circ\text{C}$ .; and 28°C. supermaintenance  $38.2 \pm .08^\circ\text{C}$ . No effect of prior environmental temperature or of plane of nutrition is revealed by these measurements. Furthermore, no significant correlation was found to exist between body temperature and basal metabolic rate.

weight. In the second comparison, feeding the rat at a supermaintenance level of nutrition, environmental temperature remaining constant, is associated with a highly significant increase in the basal metabolic rate, substantiating the findings presented in table 1. In the third comparison no significant difference could be found in the basal metabolic rate of rats exposed to temperatures of 4°C. and 28°C. when no great difference in the intake of food was simultaneously imposed. Inexplicably, the rats previously exposed to a temperature of 4°C., exhibited a significantly lower percentage of creatinine nitrogen in the endogenous urinary nitrogen, averaging 2 to 3% lower.

Thus, the basal metabolic rate was not elevated by prior exposure to a low environmental temperature unless the intake of food was increased at the low temperature in order to cover the increased energy requirements. Also, for the two groups of rats at 28°C., an average elevation in caloric intake somewhat less than that obtaining between the maintenance rats at 4°C. and at 28°C., occasioned an increase in basal metabolic rate of the same order of magnitude. The picture presented is thus consistent with the view that the effective agent in the stimulation of basal metabolism is the prior caloric intake, not the prior exposure to a low temperature.

Previously reported experiments purporting to show that exposure to low temperatures raises the basal metabolic rate in a subsequent period are probably subject to the same interpretation, since food intakes at the low temperatures employed would naturally be greater than food intakes at higher temperatures.

The excretion of endogenous nitrogen behaves differently. Here, a considerable difference in caloric intake at 28°C. (comparison II) was associated with no significant difference <sup>4</sup>.

<sup>4</sup> The average effect of a difference in the plane of nutrition on the excretion of endogenous nitrogen in the second experiment (comparison II, table 4) is diametrically opposed to that observed in the first experiment (table 2), indicating the insignificance of the latter, although the point probably needs further study. The comparatively low odds obtained in comparison III are assumed to be fortified by the high odds in comparison I and the obviously insignificant odds in comparison II.

TABLE 4

*Results of statistical analysis of the observed differences at different environmental temperatures and diet intake levels.*

GROUP COMPARISONS	MEAN OF DIFFERENCES <sup>1</sup>	t	ODDS
<b>I</b>			
<i>4°C. maintenance vs. 28°C. maintenance</i>			
Basal heat production, cal. per m <sup>2</sup> per day	-147.0	4.96	>100 to 1
Endogenous nitrogen excretion, mg. per m <sup>2</sup> per day	-371.0	5.74	>100 to 1
Total creatinine N, per cent of endogenous N, pct.	2.83	9.79	>100 to 1
Ratio of endogenous N to basal heat, mg. per cal.	-0.136	1.12	4 to 1
<b>II</b>			
<i>28°C. excess food vs. 28°C. maintenance</i>			
Basal heat production, cal. per m <sup>2</sup> per day	-161.8	4.34	>100 to 1
Endogenous nitrogen excretion, mg. per m <sup>2</sup> per day	-73.8	0.83	3 to 1
Total creatinine N, per cent of endogenous N, pct.	0.716	0.80	3 to 1
Ratio of endogenous N to basal heat, mg. per cal.	0.284	2.84	41 to 1
<b>III</b>			
<i>4°C. maintenance vs. 28°C. excess food</i>			
Basal heat production, cal. per m <sup>2</sup> per day	14.8	0.86	4 to 1
Endogenous nitrogen excretion, mg. per m <sup>2</sup> per day	-320.6	2.52	28 to 1
Total creatinine N, per cent of endogenous N, pct.	2.12	2.49	27 to 1
Ratio of endogenous N to basal heat, mg. per cal.	-0.42	3.13	49 to 1

<sup>1</sup> Student: Biometrika, vol. 6, p. 1, 1908. +, 1st group lower than 2nd group;  
-, 1st group higher than 2nd group.

in the endogenous output of nitrogen, while a difference in prior environmental temperature, whether accompanied by a large (comparison I) or by a small (comparison III) difference in caloric intake, was associated with a significant difference in the excretion of endogenous nitrogen, the greater excretion occurring at the lower temperature. The effective agent here seems to be prior environmental temperature, not prior plane of nutrition.

The constant relationship shown to exist between the endogenous nitrogen metabolism and the basal energy metabolism of adult rats by Terroine and Sorg-Matter ('27) and by Smuts ('35) was established in a purely empirical manner. It amounts to about 2.0 mg. of endogenous nitrogen per calorie of basal energy expenditure under our conditions of estimating the two components of the ratio. The absence of unequivocal experimental evidence to the contrary has permitted heretofore the assumption that this ratio was constant under usual conditions. In the experiments above reported, statistical evidence has been presented showing that the relationship is subject to variation under some conditions. The plane of nutrition, by stimulating one component (basal metabolic rate) and not the other (endogenous output of nitrogen), causes a depression of the ratio. Low environmental temperature accompanied by increased caloric intake is without effect upon the ratio, since both components are almost equally stimulated.

#### CONCLUSIONS

The normal ratio for adult rats, observed in this laboratory, of approximately 2.0 mg. of endogenous urinary nitrogen per calorie of basal heat is disturbed under the conditions studied if they operate in such a way as to affect the two members of the ratio differently.

A prior low environmental temperature elevates the endogenous level of nitrogen excretion whether or not the plane of nutrition is simultaneously raised. On the other hand, such a lowering of temperature elevates the basal metabolic rate only when accompanied by an elevation in the

plane of nutrition (caloric intake) and then only to an extent no greater than could be produced by the raised plane of nutrition itself. Hence, the ratio of endogenous nitrogen to basal heat may be unaffected in the albino rat by a lowering of the environmental temperature if the plane of nutrition (caloric intake) is increased in proportion to the increase in energy requirements. It will always be depressed in the same animal if the plane of nutrition is raised with no concomitant lowering of the environmental temperature.

A different picture may be expected in animals whose basal metabolic rate is not so sensitive to changes in the plane of nutrition as is that of the albino rat.

#### LITERATURE CITED

- ASZODI, Z. 1924 Tierische Kalorimetrie. III. Energieumsatz Kleiner Tiere bei chronischer Unterernährung. *Biochem. Zeit.*, vol. 152, pp. 472-478.
- BENEDICT, F. G., AND GRACE MACLEOD 1929 The heat production of the albino rat. II. The influence of environmental temperature, age, and sex; comparison with the basal metabolism of man. *J. Nutrition*, vol. 1, pp. 367-398.
- BRODY, S., R. C. PROCTER AND U. S. ASHWORTH 1934 Growth and development with special reference to domestic animals. XXXIV. Basal metabolism, endogenous nitrogen, creatinine and neutral sulphur excretions as functions of body weight. *Mo. Agr. Exp. Sta. Res. Bul.* 220. 40 pp.
- COLLIP, J. B., AND L. W. BILLINGSLEY 1936 The effect of temperature upon metabolism. *Transactions of the Am. Assoc. for the Study of Goiter*.  
1937 The effect of temperature upon metabolism. *Western J. Surg., Obstet. Gynecol.*, vol. 45, pp. 12-15.
- FONTAINE, R., R. GUILLEMET AND P. MANDEL 1938 Sur la répercussion des variations de la Température extérieure sur la dépense azotée endogène minimum chez le chien. *Compt. rend. soc. biol.*, vol. 128, pp. 103-106.
- GIAGA, J., AND S. GELLINEO 1930 Hypothermy and thermogenesis. *Arch. Intern. Physiol.*, vol. 32, pp. 237-250.
- GULICK, A. 1922 The influence of a beri-beri diet upon the metabolic rate of the white rat. *Proc. Am. Physiol. Soc., Am. J. Physiol.*, vol. 59, pp. 483-484.  
1924 The basal metabolism of white rats in relation to the intake of vitamin B. *Proc. Am. Physiol. Soc., Am. J. Physiol.*, vol. 68, pp. 131-132.
- HALDANE, J. 1892 A new form of apparatus for measuring the respiratory exchange of animals. *J. Physiol.*, vol. 13, pp. 419-430.
- HAMILTON, T. S. 1937 The thermogenic effect and the net energy content of rations balanced and unbalanced with respect to protein. Thesis, University of Illinois.

- HORST, K., L. B. MENDEL AND F. G. BENEDICT 1934 The influence of previous diet, growth and age upon the basal metabolism of the rat. *J. Nutrition*, vol. 8, pp. 139-162.
- LEE, M. O. 1929 Determination of the surface area of the white rat with its application to the expression of metabolic results. *Am. J. Physiol.*, vol. 89, pp. 24-33.
- MITCHELL, H. H., AND G. G. CARMAN 1926 Effect of excessive amounts of vitamin B on the basal metabolism of rats of different ages. *Am. J. Physiol.*, vol. 76, pp. 385-397.
- RING, G. C. 1936 An attempt to stimulate the thyroid gland in rats by exposure to cold. *Proc. Am. Physiol. Soc., Am. J. Physiol.*, vol. 116, p. 129.
- 1939 Thyroid stimulation by cold: Including the effect of changes in body temperature upon basal metabolism. *Am. J. Physiol.*, vol. 125, pp. 244-250.
- SCHWABE, E. L., F. E. EMERY AND F. R. GRIFFITH, JR. 1938 The effect of prolonged exposure to low temperature on the basal metabolism of the rat. *J. Nutrition*, vol. 15, pp. 199-210.
- SCHWABE, E. L., AND F. R. GRIFFITH, JR. 1936 The effect of exposure to cold upon the basal metabolism of the rat. *Proc. Am. Physiol. Soc., Am. J. Physiol.*, vol. 116, p. 140.
- SMUTS, D. B. 1935 The relation between the basal metabolism and the endogenous nitrogen metabolism with particular reference to the estimation of the maintenance requirement of protein. *J. Nutrition*, vol. 9, pp. 403-433.
- TERROINE, E. F., AND H. SORG-MATTER 1927 Loi quantitative de la depense azotée minima des homeothermes: Validité interspecifique. *Arch. Intern. Physiol.*, vol. 29, pp. 121-132.
- 1928 Influence de la température extérieure sur la dépense azotée endogène des homeothermes: Validité interspecifique. *Arch. Intern. Physiol.*, vol. 30, pp. 115-125.
- WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. *Science*, vol. 75, pp. 339-340.



# THE PATHOLOGY OF RIBOFLAVIN DEFICIENCY IN THE RAT<sup>1</sup>

JAMES H. SHAW AND PAUL H. PHILLIPS

*Department of Biochemistry, College of Agriculture, University of Wisconsin,  
Madison*

## SIXTEEN FIGURES

(Received for publication June 5, 1941)

## INTRODUCTION

Phillips and Engel ('38) have shown in the chick that a ration low in riboflavin results in a specific neuropathology of the main peripheral nerve trunks characterized by degenerative changes in the myelin of the nerve fiber, accompanied by Schwann cell proliferation, axis cylinder swelling and fragmentation. In rats on the same ration, Engel and Phillips ('39) were unable to demonstrate any similar changes. Wolbach ('37) had previously stated that there was no such degeneration in the rat on a vitamin B<sub>2</sub> low ration. In the dog he was able to demonstrate degeneration of the myelin sheaths in the peripheral nerves and in the posterior tracts of the spinal cord.

The occurrence of cataract in albino rats on a ration low in riboflavin has been described by various workers. Day, Darby and Langston ('37) and Day, Darby and Cosgrove ('38) showed the identity of riboflavin with the cataract preventive factor and the arrest of early nutritional cataract by the use of riboflavin.

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

These studies were made possible by grants from the Wisconsin Alumni Research Foundation and the Works Progress Administration.

Recent investigations by Wagner, Axelrod, Lipton and Elvehjem ('40) and Mannerling, Lipton and Elvehjem ('41) on the riboflavin requirement of the rat on synthetic rations have indicated that the riboflavin requirement of the rat is considerably greater on a ration high in fat than on a ration high in carbohydrate. On high fat rations, they were able to produce a more severe riboflavin deficiency characterized by spastic paralysis. A thorough investigation of the pathology of riboflavin deficiency in the rat on both carbohydrate and high fat rations which were strictly riboflavin deficient was undertaken to determine if there was any neuropathology or general pathology in the albino rat.

#### EXPERIMENTAL PROCEDURE

Two rations were used which were deficient in riboflavin. The first was a modification of ration K<sub>21</sub> described by Wagner, Axelrod, Lipton and Elvehjem ('40) and consisted of the following:

	%
Dextrin	72
Alcohol ext. casein	18
Lard	3
Corn oil	3
Salts IV	4
	100
PER 100 GM. RATION	
Thiamine	300 µg.
Pyridoxine	300 µg.
Nicotinic acid	5 mg.
Pantothenic acid	2 mg.
Choline	200 mg.

The second ration was identical with the first with the exception that 49.5 parts of the dextrin were replaced isocalorically by lard. The fat content of this ration was 39%. Twice weekly, these rations were further supplemented by feeding 3 drops of haliver oil which contained 1 mg. of added synthetic  $\alpha$ -tocopherol per 3 drop dose. Small quantities of ration were mixed as needed. The rations were stored in the refrigerator and fed daily to prevent rancidity.

Male rats ranging in weight from 60-80 gm. were distributed among four lots as follows:

LOT NO.	NO. OF RATS	PERIOD FED (IN WEEKS)	RATION
I (a)	5	8	Basal carbohydrate ration only
(b)	5	12	Basal carbohydrate ration only
II (a)	5	8	Basal carbohydrate ration ad libitum, plus 100 µg. riboflavin per day
(b)	5	12	Basal carbohydrate ration, intake restricted to that of individual rats in lot I (b), plus 100 µg. riboflavin per day
III (a)	5	4	Basal high fat ration only
(b)	13	8	Basal high fat ration only
IV (a)	5	4	Basal high fat ration ad libitum, plus 100 µg. riboflavin per day
(b)	13	8	Basal high fat ration intake restricted to that of individual rats in lot III (b), plus 100 µg. riboflavin per day

Group (a) in each lot was sacrificed early to study the progress of the pathology. The rats in group (b) of lots I and III were on experiment until severe deficiency symptoms appeared. When a riboflavin deficient rat became feeble, it was killed and autopsied. At the same time its mate in the control group was killed and a similar autopsy performed. Routine histological examinations were made of the adrenal, pancreas, testis, kidney, liver, spleen, pituitary, thyroid, thymus, stomach and small intestine, after they had been fixed in Bouin's fluid and stained in hematoxylin and eosin. Studies of the nervous system were made on the brain, the cervical and brachial segments of the spinal cord, the sciatic and inferior branch of the brachial plexus. The myelin sheaths of the sciatic and inferior branch of the brachial plexus were studied by means of plane polarized light between crossed nicol prisms (Setterfield and Sutton, '35) and by the Marchi osmic acid technique (Swank and Davenport, '34) after fixation in 10% formalin buffered at pH 7.0. The myelination of the cervical region of the cord was also studied by the Marchi technique. The axis cylinders of the brachial segment of the cord and the peripheral nerves were observed after silver impregnation by the Bodian method ('36). Nissl

preparations of the spinal cord and brain were made with gallocyanin according to Einarson's method ('32). All the tissues studied by either Bodian's silver or Einarson's gallo-cyanin methods had been previously fixed in a fluid consisting of 100 parts of 95% alcohol, 5 parts of glacial acetic acid and 2 parts of paraldehyde.

### RESULTS

The rates of growth of the rats used in this experiment are shown in figure 1. It can be seen that when the rats were allowed access to the basal rations supplemented daily with riboflavin, good growth resulted. When the food intake of

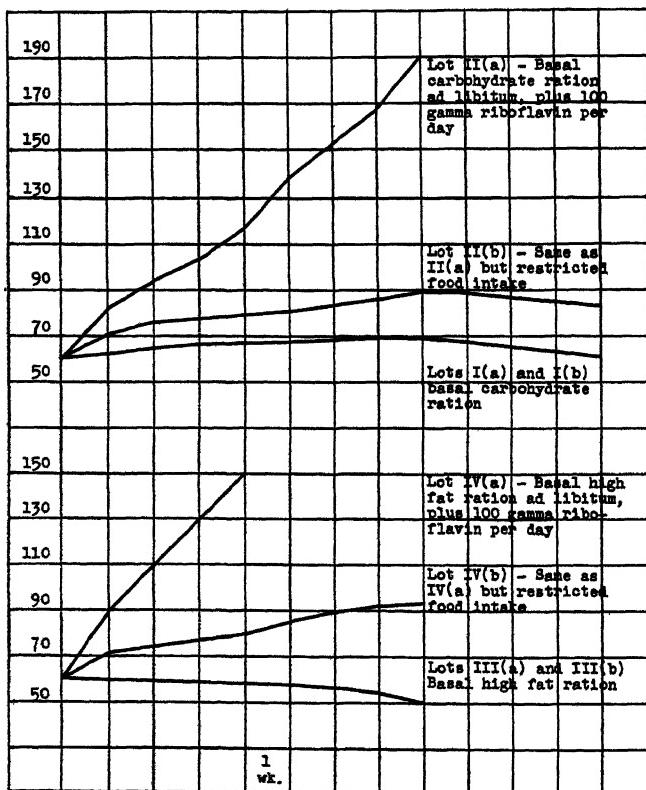


Fig. 1 Growth curves for rats raised on the carbohydrate and high fat rations.

the positive controls was restricted to the level of the riboflavin deficient rats, growth was considerably retarded.

The hair coat of the rats on the basal carbohydrate ration became rough and unkempt with a slight loss of hair. There was some dermatitis about the nose. In contrast, the rats on the high fat ration lost large amounts of hair so that sizable areas, particularly between the legs, under the belly and around the face, became almost completely denuded. The remaining hair was heavily matted together with lumps of the greasy ration. Dermatitis was much more severe in these animals. Considerable areas around the nose became necrotic and raw. In addition these rats developed a parchment-like skin and frequently, where it was wrinkled or folded, heavily encrusted ulcers. A slight hemorrhagic inflammation of the nasal mucous membranes of the deficient rats was common and resulted in a crusting of blood around the nostrils. The eyelids became sticky and there was noted a marked decrease in the amount of lacrimation which left the cornea quite dry at all times. Frequently both eyelids would stick together and the eyes would remain closed for several days. The eyes appeared to be swollen and slightly red. On the carbohydrate ration two cases of cataract developed. However six of the rats on the high fat ration developed a corneal opacity in one or both eyes.

With one exception locomotion was normal in all the rats on the basal carbohydrate ration. In the exceptional animal, a spastic gait developed which was also seen in eleven of the animals on the high fat ration. The remainder of the animals became weak and feeble without any gross evidence of a neurological disturbance (fig. 2). In those rats which developed a spastic gait, the hind legs became very stiff and were rigidly extended. Locomotion was very difficult due to the inability of the animal to flex the hind legs. Walking was accompanied by a side-to-side swaying motion produced when the body weight was shifted entirely to one leg while the opposite one was swung forward. Wherever the rat survived this spastic gait period for 2 or 3 days, the front legs ex-



Fig. 2 The rat on the right was fed the low riboflavin carbohydrate ration for 12 weeks. The rat on the left received the same ration with intake restricted to that of the former plus 100 µg. riboflavin per day.  
Fig. 3 The rat on the right was fed on the high fat ration for 8 weeks. The rat on the left received the same ration with intake restricted to that of the former plus 100 µg. riboflavin per day.

hibited the same stiffness. The back of these rats was highly arched (fig. 3). The deficient animals were extremely restless. Even when spastic paralysis was severe, there was no attempt to rest. There was a slight increase in the food consumption during the latter stages of this type of paralysis.

The time of the onset of the severe symptoms varied by a period of several weeks on the two types of rations. Invariably the rats on the high fat ration began to show severe deficiency symptoms at the end of about 8 weeks, while those on the carbohydrate ration did not show severe symptoms before the end of 12 weeks.

The terminal stage in all these cases was a deep coma and death. In coma those which did not exhibit spastic paralysis were limp as though anesthetized, and the limbs of those with spastic paralysis were stiffly outstretched.

The rats on the basal rations when supplemented daily with 100 µg. riboflavin were normal in all respects.

The distribution of histological changes in the tissues of these rats is shown by table 1.

TABLE 1

In those animals removed from experiment before paralysis had developed no neuropathology could be demonstrated by the techniques used. However, in those animals where paralysis was apparent a degeneration of the myelin of a few of the fibers of the sciatic could be demonstrated (figs. 5 and 7). In the early stages the inferior branch of the brachial plexus and the spinal cord were normal. When spastic paralysis became more severe, degeneration of the sciatic became very widespread with very few of the fibers remaining normal (fig. 6). At this stage a positive Marchi reaction was obtained in the cervical region of the spinal cord. This seemed to be localized mainly in the dorsal regions of the cord (figs. 8 and 9). Degeneration was also seen in the inferior branch of the brachial plexus. Where degeneration of the myelin sheaths had taken place in the sciatic and inferior branch of the brachial plexus fragmentation and bulbous swelling of the fibers could be shown with silver impregnation. In the regions of the cord where it was severely affected by these processes gliosis occurred. In the mild cases, there was only a slight positive Marchi reaction in the spinal cord unaccompanied by any repair process.

The testis in all the riboflavin deficient rats was atrophied, rather firm but retracted into the body cavity. This atrophy developed in the rats fed the basal rations for periods of 4 to 6 weeks. The epididymus was proportionately smaller and the prostate and seminal vesicles were grossly atrophied.

---

Fig. 4 Sciatic nerve. High fat ration plus riboflavin. Normal myelination. Marchi.  $\times 440$ .

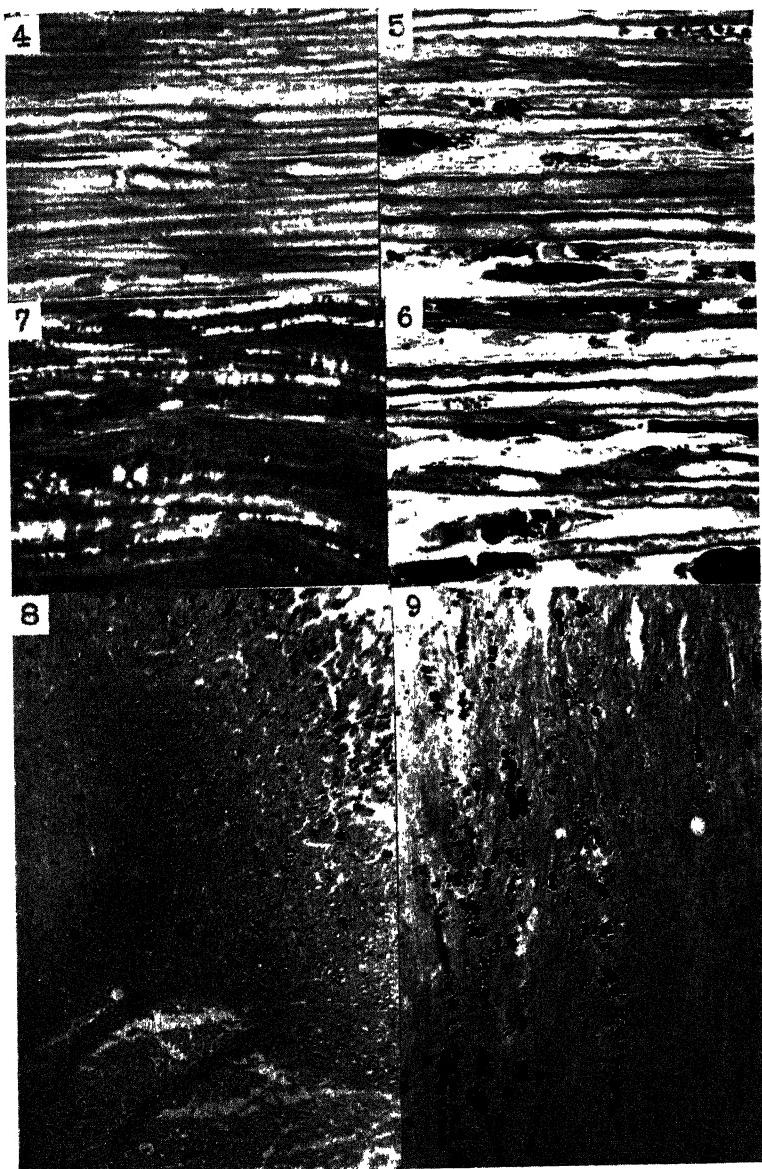
Fig. 5 Sciatic nerve. High fat ration only. Mild myelin degeneration in a few of the fibers. Marchi.  $\times 440$ .

Fig. 6 Sciatic nerve. High fat ration only. Widespread myelin degeneration. Marchi.  $\times 440$ .

Fig. 7 Sciatic nerve. High fat ration only. Degeneration of myelin as shown by disturbed distribution of birefringent materials in the myelin sheath. Crossed nicols and polarizing microscope.  $\times 600$ .

Fig. 8 Transverse section of spinal cord. High fat ration only. Myelin degeneration in the dorsal paths of the cord. Marchi.  $\times 200$ .

Fig. 9 Longitudinal section of spinal cord. High fat ration only. Myelin degeneration in the dorsal paths of the cord. Marchi.  $\times 200$ .



Figures 4 to 9.

On histological examination of the testis, the seminiferous tubules were either undeveloped or atrophied. The lumina were much larger in relation to the diameter of the tubules than they were in the normal testis. In the early stages there was no evidence of spermatids, and there was a decrease in the number of meiotic divisions (fig. 11). Later the spermato-  
gonial cells were clumped in the lumen of the tubule leaving the Sertoli cells lying bare against the basement membrane (fig. 12). The lumina of the tubules were commonly filled with detached spermatogonia in all stages of arrested development and degeneration. The epithelial cells of the tubules of the epididymus and their stereocilia appeared to be normal. The lumina of these tubules were filled with spermatogonia but no spermatozoa were seen.

The thymus in the riboflavin deficient rat had undergone an early involution which apparently had followed the normal course of aging. There was a decrease in the number of lymphocytes in the cortical region and then a replacement of these cells with adipose tissue (fig. 14). In the most chronic deficiency produced on the high fat ration the thymus had undergone complete involution and consisted of only a few lymphocytic cells surrounding the scattered Hassall's bodies.

In a few of the more severe cases the thyroid gland in the riboflavin deficient rat contained much less colloid than that of the control rats (fig. 16). The follicular epithelial cells

---

Fig. 10 Testis. High fat ration plus riboflavin. Normal tubules with meiotic divisions and maturing spermatids. H and E.  $\times 440$ .

Fig. 11 Testis. High fat ration only. Early degenerative changes with decrease in the number of meiotic divisions and complete lack of spermatids H and E.  $\times 440$ .

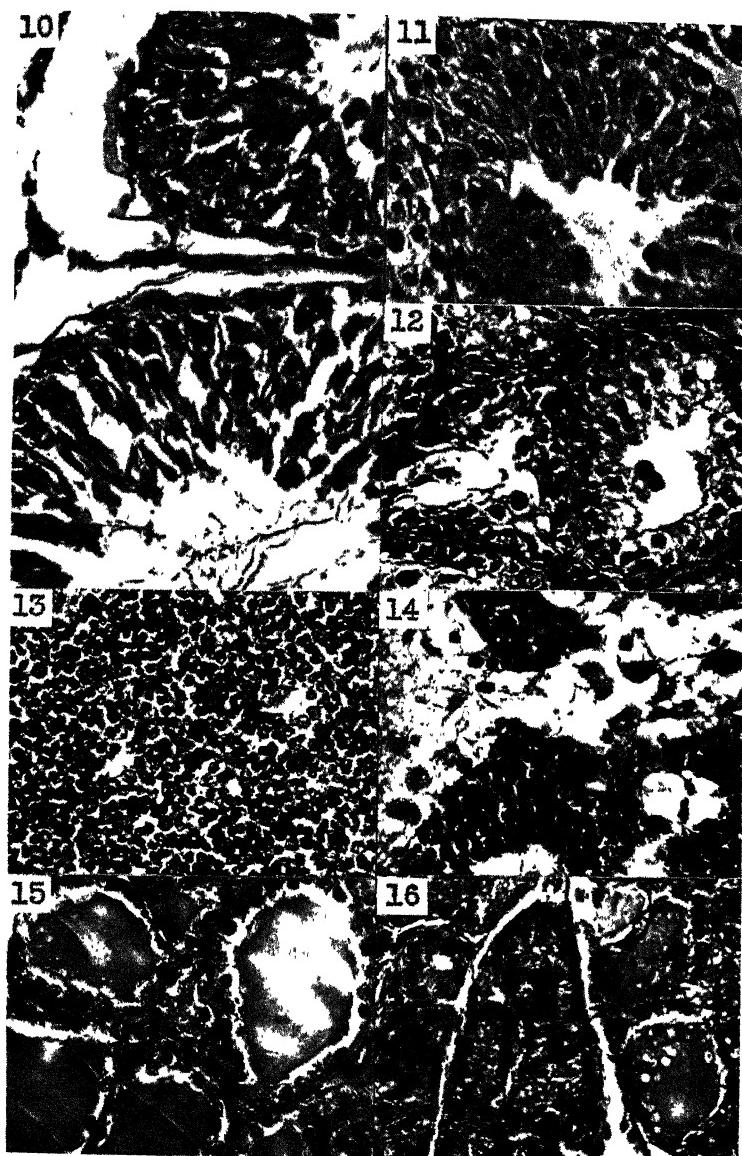
Fig. 12 Testis. High fat ration only. Late degenerative changes with complete disappearance of meiotic divisions, shrinkage of tubules. H and E.  $\times 440$ .

Fig. 13 Thymus. Carbohydrate ration plus riboflavin. Normal number and distribution of lymphocytes. H and E.  $\times 440$ .

Fig. 14 Thymus. Carbohydrate ration only. Decrease in number of lymphocytes and infiltration of adipose tissue. H and E.  $\times 440$ .

Fig. 15 Thyroid. High fat ration plus riboflavin. Normal low cuboidal epithelial cells in acini with large colloid filled lumina. H and E.  $\times 440$ .

Fig. 16 Thyroid. High fat ration only. High cuboidal epithelial cells in acini with reduced lumina and decreased amount of colloid. H and E.  $\times 440$ .



Figures 10 to 16.

had undergone hypertrophy. The normal low cuboidal actively secreting epithelial cells were replaced by high cuboidal epithelial cells.

The adrenal in severe cases was also somewhat affected. The cells of the actively secreting area (*zona fasciculata*) of the cortex were scattered and diffuse in nature so that the cells did not lie in organized cords but resembled the distribution of cells in the post-secretory area (*zona reticulata*).

In all cases, the pancreas, kidney, liver, spleen, pituitary, stomach and small intestine appeared normal.

#### DISCUSSION

The deficiency of riboflavin in the ration of the rat produced a variety of pathological changes. The time of appearance and the severity of these changes varied depending on the type of ration used. When a carbohydrate ration deficient in riboflavin was fed, the symptoms appeared later and were much less severe than when this diet was replaced by an isocalorically equivalent high fat — low riboflavin ration. This fact has already been reported by Mannerling et al. ('41).

These results demonstrate clearly that a riboflavin deficiency produced on a strictly deficient diet causes neuropathy in the rat similar to that in the chick (Phillips and Engel, '38). This probably explains the results previously obtained by Engel and Phillips ('39) who reported no histopathology in the nervous system of the rat fed a low riboflavin diet. In addition to the changes in the nervous system it is quite apparent that other structures are damaged early in the development of a riboflavin deficiency in the rat. The thymus, thyroid, and testis show histologic changes earlier than neural lesions. It would appear that riboflavin is utilized by the body at the expense of these tissues.

It must be kept in mind that these histopathologic changes produced on riboflavin deficient rations may be secondary in nature but at least they are always associated with the histopathology of this deficiency.

These researches tend to emphasize again the interrelationship of riboflavin and lipid metabolism, a fact which is supported by the work of Phillips and Engel ('38) and Mannerling, Lipton and Elvehjem ('41). The demonstration that such cellular structures as the testis and thymus are affected is suggestive of a more intimate relationship with cellular metabolism. This might well be concerned with phosphorylation as suggested by Mannerling et al. ('41).

#### SUMMARY

It has been demonstrated that a severe riboflavin deficiency in the rat results in a partial paralysis of the legs. This type of paralysis is produced more easily on a high fat ration and it is prevented by the daily feeding of crystalline riboflavin. This paralysis in its severe form is characterized by degeneration of the myelin sheaths of the nerves accompanied by axis cylinder swelling and fragmentation. Myelin degeneration and gliosis in the spinal cord have been observed.

Histologic examination of the testis and thymus of riboflavin deficient rats shows that there is an early and marked atrophy of the testis and an abnormally early involution of the thymus. Structural changes are also observed in the thyroid and adrenal of the most severe cases. These changes have been described.

It is concluded from these studies that a riboflavin deficiency in the rat causes histopathologic changes in certain endocrine glands, the testis and also in the central nervous system.

#### LITERATURE CITED

- BODIAN, D. 1936 A new method for staining nerve fibers and nerve endings in mounted paraffin sections. *Anat. Rec.*, vol. 65, p. 89.
- DAY, P. L., W. J. DARBY AND W. C. LANGSTON 1937 The identity of flavin with the cataract preventive factor. *J. Nutrition*, vol. 13, p. 389.
- DAY, P. L., W. J. DARBY AND K. W. COSGROVE 1938 The arrest of nutritional cataract by the use of riboflavin. *J. Nutrition*, vol. 15, p. 83.
- EINARSON, L. 1932 A method for progressive staining of Nissl and nuclear substance in nerve cells. *Am. J. Path.*, vol. 8, p. 295.
- ENGEL, R. W., AND P. H. PHILLIPS 1939 Effect of riboflavin-low diets upon nerves, growth and reproduction in the rat. *Proc. Soc. Exp. Biol. Med.*, vol. 40, p. 597.

- MANNERING, G. J., M. A. LIPTON AND C. A. ELVEHJEM 1941 Relation of dietary fat to riboflavin requirement of growing rats. Proc. Soc. Exp. Biol. Med., vol. 46, p. 100.
- PHILLIPS, P. H., AND R. W. ENGEL 1938 The histopathology of neuromalacia and "curled toe" paralysis in the chick fed low riboflavin diets. J. Nutrition, vol. 16, p. 451.
- SETTERFIELD, H. E., AND T. S. SUTTON 1935 The use of polarized light in the study of myelin degeneration. Anat. Rec., vol. 61, p. 397.
- SWANK, R. L., AND H. A. DAVENPORT 1934 Marchi's staining method: Studies of some of the underlying mechanisms involved. Stain Tech., vol. 9, p. 11.
- WAGNER, J. R., A. E. AXELROD, M. A. LIPTON AND C. A. ELVEHJEM 1940 A rat assay method for the determination of riboflavin. J. Biol. Chem., vol. 136, p. 357.
- WOLBACH, S. B. 1937 Pathologic changes resulting from vitamin deficiency. J. Am. Med. Assoc., vol. 108, p. 7.

## VITAMIN B COMPLEX AND FAT METABOLISM

J. C. FORBES

*Department of Biochemistry, Medical College of Virginia,  
Richmond, Virginia*

(Received for publication June 26, 1941)

McHenry and Gavin ('40) showed that the administration of a beef liver concentrate to rats on a fat-free diet, containing adequate amounts of thiamine, riboflavin and pyridoxine, led to a marked increase in liver fat and cholesterol which was prevented by lipocaiac, but not choline. Recently these authors have reported that the addition of pantothenic acid to the vitamin supplement was not sufficient to produce the choline resistant fatty liver but, if biotin was given in addition, choline-resistant fatty livers were produced ('41).

Studies on the effect of the B complex vitamins on fat metabolism have been in progress in this laboratory for over a year and the results obtained with pantothenic acid are in complete agreement with those of McHenry and Gavin. However, contrary to the observations of Gavin and McHenry ('40) we have found that the administration of nicotinic acid renders the liver lipids somewhat more resistant to the lipotropic action of choline. Also, under the conditions studied, the administration of this vitamin definitely increases the cholesterol content of the liver. The experimental conditions differed, however, from the work of the above authors with nicotinic acid in that our diet supplied adequate amounts of pantothenic acid while theirs contained none. This difference in supplements may account for the difference in results.<sup>1</sup>

Young rats of both sexes, weighing from 70 to 90 gm., were used. All analyses were made on individual livers, but as no

<sup>1</sup> The vitamin preparations used were kindly supplied by Merck and Company.

difference was found between the sexes, the results are pooled irrespective of sex in the accompanying tables. All of the animals were put on one of the basal diets given in table 1 for a period of 2 or 3 weeks before addition of the supplements.

TABLE 1  
*Basal diets*

	DIET 1 gm.	DIET 2 gm.
Casein, vitamin free, Labco	10	10
Sucrose	83	83
Salt mixture	4	4
Agar	2	2
Cod liver oil	1	—
Porcomorph oil	—	0.03
Supplemented by		
Thiamine chloride	0.2 mg.	0.2 mg.

The methods employed for the liver analyses were the same as described previously by Outhouse and Forbes ('40). For the determination of body lipids, the intestinal tract, liver, skin, feet and tail were removed and the carcass ground in a meat grinder. After grinding, the carcasses were weighed, placed in an oven at approximately 100°C. and dried for several days. The material was then reweighed, ground finely in a mortar and samples taken for analysis. The weighed sample, usually about 1 gm., was again ground with approximately 5 gm. of a commercial zeolite,<sup>2</sup> transferred to a 125 cc. glass-stoppered bottle and 100 cc. of chloroform added. It was then set aside with occasional shaking for at least 24 hours, after which aliquots were taken for analysis. All livers were analyzed separately, but the bodies were usually run in groups of two to four. It would be preferable to determine the lipid content of the whole body but it was found impossible to obtain satisfactory samples for analysis if the skin was not removed before the bodies were ground. However, since the same procedure was used in all of the experiments, the results

<sup>2</sup> Doucil. Material used in fat analyses was obtained from W. A. Taylor and Company, Baltimore, Maryland.

are comparable. In two sets of experiments the lipid content of the dried skin was determined. It was found that in each of the experiments the neutral fat plus cholesterol content varied more or less directly with that of the rest of the body. Consequently, it seems likely that the same general relationship in lipid values would have been obtained if the whole body had been used for analysis.

In experiments 6 and 7, in order to eliminate fat completely from the diet, the vitamin A and D preparation was discontinued during the feeding of supplements. In experiments 11, 12 and 14, 50% porcomorph oil<sup>3</sup> was used to the extent of 0.03% of the diet throughout the experimental period. Since *L*-cystine is known to increase the deposition of fat in the livers of rats on a high fat-low choline diet, it was decided to add this amino acid to the fat-free diet, in certain of the experiments, in the hope that under these conditions it might favor the deposition of fat in the livers of the experimental animals. Consequently in experiments 5, 6 and 7 the diet included 0.5% cystine during the supplement feeding period. In experiments 11 and 12 the diet included 0.1% cystine throughout the experimental period. In all experiments the vitamin supplements, when fed, were given at the following levels, pyridoxine 500 µg., riboflavin 500 µg., calcium pantothenate 1600 µg. and nicotinic acid 1600 µg. per 100 gm. of food. In experiments 4, 5, 6 and 7 the choline-fed rats received 10 mg. of choline chloride per rat per day. In experiments 12 and 14 choline chloride was incorporated in the diet to the extent of 10 mg. per 10 gm. of food, and the average food consumption exceeded 10 gm. per day.

#### DISCUSSION

The experimental results recorded in table 2 show that the administration of either pyridoxine, riboflavin or calcium pantothenate, in addition to thiamine, exerts no demonstrable effect upon the concentration of neutral fat or cholesterol in the liver, over that produced by thiamine alone. That pyridoxine has no effect, was demonstrated previously by Gavin

<sup>3</sup> Mead Johnson.

TABLE II  
*Effect of various supplements on the cholesterol and neutral fat content of the body and liver.*

EXPERIMENTAL NO.	NUM. OF RATS	AVERAGE DAILY GAIN IN WEIGHT DURING SUPPLEMENT FEEDING PERIOD	LAYER			MINCED BODY TISSUE			SUPPLEMENTS
			Neutral fat	Chole-	Neutral	Chole-	Neutral	Chole-	
11	5	gm.	%	gm.	%	gm.	%	gm.	
	6	-0.7	10.4	0.42	0.538	0.022	3.12	0.18	31 days on diet 2.
		+0.1	10.9	0.45	0.556	0.023	3.10	0.20	10 days on Ca pantothenate in addition.
4	4	-0.4	13.0	0.46	0.619	0.023	3.31	0.19	21 days on diet 2.
	3	-0.2	11.6	0.35	0.620	0.031	3.98	0.17	21 days on diet 2.
	4	+1.1	16.4	0.34	0.995	0.021	5.82	0.18	21 days on diet 1. 10 days on B <sub>2</sub> , B <sub>6</sub> and Ca pantothenate in addition.
	5	+1.2	3.7	0.26	0.160	0.012	7.62	0.18	Same plus choline during suppl. feeding period.
	3	+0.2	5.4	0.22	0.204	0.010	3.89	0.21	21 days on diet 1. 10 days on B <sub>2</sub> and B <sub>6</sub> in addition.
	5	+0.5	1.0	0.17	0.043	0.007	5.30	0.18	Same as above plus choline during suppl. feeding period.
	4	+0.9	25.1	0.65	1.666	0.045	4.22	0.18	21 days on diet 1. 10 days on B <sub>2</sub> , B <sub>6</sub> and Ca pantothenate in addition.
	5	+1.8	5.0	0.41	0.204	0.018	5.31	0.19	Same as above plus choline during suppl. feeding period.
	5	+0.8	18.6	0.38	1.045	0.019	4.22	0.18	21 days on diet 1. 10 days on B <sub>2</sub> and B <sub>6</sub> in addition.
	5	+1.3	6.8	0.30	0.314	0.014	4.41	0.19	Same as above plus choline during suppl. feeding period.
	6	+1.3	25.9	0.47	1.922	0.035	3.51	0.19	14 days on diet 2. 9 days on B <sub>2</sub> , B <sub>6</sub> and Ca pantothenate in addition.
	5	+1.5	2.7	0.24	0.151	0.014	3.80	0.20	Same as above plus choline during suppl. feeding period.
	5	+2.0	16.5	0.38	0.959	0.019	3.03	0.17	14 days on diet 2. 9 days on B <sub>2</sub> , B <sub>6</sub> and Ca pantothenate in addition.
	5	+1.6	2.6	0.19	0.118	0.009	3.40	0.20	Same as above plus choline during suppl. feeding period.
	7	+1.0	18.7	0.66	1.001	0.037	5.21	0.19	14 days on diet 2. 11 days on B <sub>2</sub> , B <sub>6</sub> and Ca pantothenate in addition.
	5	+0.4	1.5	0.25	0.062	0.010	3.05	0.15	Same as above plus choline during suppl. feeding period.
	5	-0.7	4.9	0.38	0.175	0.015	1.61	0.19	25 days on diet 2. No A or D during last 11 days.
	5	-0.8	1.0	0.31	0.035	0.011	1.50	0.20	14 days on diet 2. 11 days on Ca pantothenate without B <sub>6</sub> . Same as above plus choline during suppl. feeding period.
	12	+2.9	22.2	1.47	1.511	0.098	6.83	0.17	21 days on diet 2. 10 days on B <sub>2</sub> , B <sub>6</sub> and Ca pantothenate and niacinic acid in addition.
	9	+3.2	6.4	0.77	0.305	0.036	7.23	0.17	Same as above plus choline during suppl. feeding period.
	5	+1.3	11.8	0.75	0.659	0.042	5.80	0.22	21 days on diet 2. 10 days on B <sub>2</sub> , B <sub>6</sub> and Ca pantothenate in addition.
	7	+1.4	15.7	1.53	0.875	0.085	6.10	0.25	21 days on diet 2. 10 days on B <sub>2</sub> , B <sub>6</sub> and Ca pantothenate and niacinic acid in addition.
	5	+2.0	5.6	0.62	0.275	0.030	7.80	0.23	Same as above plus choline during suppl. feeding period.

<sup>1</sup> If the value for one animal which grew very little is excluded, this value becomes 1.7.

and McHenry ('40). The present investigation shows that the administration of both riboflavin and pyridoxine increased as a rule the neutral fat content of the liver but exerted no definite effect upon the concentration of liver cholesterol. When, in addition, calcium pantothenate was given, the neutral fat content was further increased, and at the same time the concentration of liver cholesterol was also raised. When nicotinic acid was given in addition to these, the effect on liver cholesterol was very striking, the total amount in the liver being in some cases increased to about ten times the normal value. On the other hand, the concentration of neutral fat was not materially increased over that obtained when thiamine, riboflavin, pyridoxine and calcium pantothenate only were given. The body neutral fat content, however, was definitely elevated over that obtained in the absence of nicotinic acid. Choline administration exerted a definite lipotropic action under all conditions studied. On the whole, however, this effect was definitely less marked in those animals receiving nicotinic acid. The feeding of choline also reduced the deposition of cholesterol in the livers. In the case of the nicotinic acid-fed animals, however, the liver cholesterol values remained quite high, on the average, in spite of choline administration. These results suggest that nicotinic acid might be a factor in the production of choline-resistant fatty livers. However, since Gavin and McHenry ('40) have shown previously that choline will prevent fatty livers when the animals are fed a diet containing nicotinic acid, thiamine, riboflavin and pyridoxine but no pantothenic acid, it would appear that no particular factor of the vitamin B complex is directly responsible. It is probable that all of them may influence the final result. It may be significant that in all experiments in which choline-resistant fatty livers have been reported the rate of growth of the animals during the supplement feeding period was quite rapid (McHenry and Gavin, '40, '41; Engel and Phillips, '39). Since a rapid rate of growth is usually associated with the consumption of a large amount of food, it is possible that a fairly large carbohydrate intake is one of the essential factors in the production of choline-resistant fatty livers.

## SUMMARY

The administration of thiamine, riboflavin, pyridoxine, nicotinic acid and calcium pantothenate to rats on a fat-free, 10% casein, high carbohydrate diet led to the production of fatty livers containing quite a high concentration of cholesterol. When nicotinic acid was absent from this diet the animals developed fatty livers whose cholesterol content, though elevated, was low in comparison with that obtained when nicotinic acid was also fed. Addition of choline to the diet exerted a definite lipotropic action under all conditions studied. However, its effect as a whole was less pronounced in those animals receiving nicotinic acid than in the others.

## LITERATURE CITED

- ENGEL, R. W., AND P. H. PHILLIPS 1939 Fatty livers as a result of thiamine administration in vitamin B<sub>1</sub> deficiency of the rat and chick. *J. Nutrition*, vol. 18, p. 329.
- GAVIN, G., AND E. W. McHENRY 1940 The B vitamins and fat metabolism. *J. Biol. Chem.*, vol. 132, p. 41.
- MCHENRY, E. W., AND G. GAVIN 1940 The effect of liver and pancreas extracts upon fat synthesis and metabolism. *J. Biol. Chem.*, vol. 134, p. 683.
- 1941 The effect of biotin upon the synthesis of lipids in rats. *Proc. Am. Soc. Biol. Chem.* *J. Biol. Chem.*, vol. 140, p. lxxxvii.
- OUTHOUSE, E. L., AND J. C. FORBES 1940 A micromethod for the determination of tissue lipids. *J. Lab. and Clin. Med.*, vol. 25, p. 1157.

# DIETARY PRODUCTION OF CATARACTS IN LARVAL AMBLYSTOMA TIGRINUM

ESTHER M. PATCH

*Department of Physiology, University of Wisconsin Medical School, Madison*

ONE PLATE (NINE FIGURES)

(Received for publication May 19, 1941)

The small vertebrates of the genus *Ambystoma* have been used extensively in analyses of various general principles of early vertebrate morphology and physiology. The author has found a striking resemblance between the dietary responses of the rapidly-growing larvae of *A. tigrinum* and those of higher vertebrates. The larvae are highly sensitive to dietary factors influencing growth and development. In addition, diseases common to man are easily produced in the small urodeles by the same dietary irregularities causing those diseases in the human form. Faulty calcification, tetany, anemia, and cataracts have resulted from different dietary inadequacies. Two types of lens changes have appeared (Patch, '34, '41), and these have consistently followed use of diets differing in the protein ingredient, the chief food requirement of these carnivores. Because of this specific relation and also the similarity between the lens changes in the salamander larvae and in man, these animals are excellent subjects for studying etiology of the disease.

## A. NORMAL LENSES IN ANIMALS FED BEEF MUSCLE DIET

A diet with powdered beef "round" as chief component, serving as a calcium-low basis for the study of needs of

salamander larvae for the calcifying minerals,<sup>1</sup> produced normal lenses and hence served as a control diet in the study of cataract formation. The basal ration contained powdered beef (63%), fat<sup>2</sup> (5%), cod liver oil (2%), bakers' yeast (1%), and cornstarch (29%) cooked into a paste for binding the ration (Hubbell and Mendel, '27). In the first experiment recorded here ('33), the beef ration was fed to forty-six larvae of the species *A. tigrinum* raised from eggs of an Illinois strain. The animals were in five groups given different combinations of calcium, phosphorus, and other mineral nutrients. In each of these groups the eyes were normal, the lenses appearing black by reflected light (fig. 1), colorless and transparent by transmitted light. No lens disturbance could be detected either by binocular examination or by histological methods. This diet produced good growth and an adequate blood supply and, when properly supplemented, led to development through normal metamorphosis to the adult form.

#### B. CATARACTS CAUSED BY A DIET OF PURIFIED CASEIN AND POWDERED MILK

A diet of casein and powdered milk (table 1) was used to determine whether the anemia previously observed in salamander larvae fed this diet (Patch, in press) was due to deficiency of iron and copper as had been found true of the widely-studied milk-anemia of rats. In order to have a minimum of minerals in the basal ration the chief ingredient, casein,<sup>3</sup> was carefully prepared. This protein, separated from

<sup>1</sup> The content of the foodstuffs, minerals and vitamins used in the rations for the salamander larvae was determined by their response in preliminary experiments with different proportions of those materials (Patch, in press), and by reference to analyses of the natural early diet of the larvae which consists largely of Entomostraca. The plant-characteristic vitamin C was not given to the flesh-eating salamanders. "Maximum feedings" were given, as used by Springer ('09) in growth studies of the newt, and in correspondence with the "ad libitum" feeding of experimental rats. All groups of animals in a series of comparisons were fed by the same time schedule.

<sup>2</sup> Wesson oil.

<sup>3</sup> W. R. Todd supplied the purified casein used in 1933 and 1934. Frank Stirn and Edwin Hove supplied that used in 1935 and 1936.

skim milk, was given seven or more alternations of solution by dilute alkali and precipitation by acid (Todd, Elvehjem and Hart, '34; Hubbell and Mendel, '27). The diet was given to forty-two *A. tigrinum* larvae of the same lot as those fed the muscle diet. The animals were divided into six groups, each with a different combination of iron and copper levels.

Before the end of 2 weeks, growth of these larvae had lagged behind that of those fed the muscle ration, and during the seventh week those fed the casein showed a poor appetite and a weakness of movement. Eleven had died before it was realized that the low food intake was partly due to poor vision caused by clouding of the lens. During the following 5 weeks,

TABLE 1  
*Composition of the casein ration.*

INGREDIENTS	PROTEIN	CARBO-HYDRATE	FAT	MINERALS	VITAMINS	TOTAL
	%	%	%	%		%
Casein	45	..	..	..	..	45
Whole milk powder	10	14	11	2	A, B, G	37
Cornstarch	..	15	..	..	..	15
Cod liver oil	..	..	2	..	A, D	2
Yeast powder	1	..	..	..	B, G	1
Total	56	29	13	2		100

eleven cases of cataract (36%) developed in the thirty-one surviving animals. Increase of both vitamin supplements failed to improve the health of the larvae. Seven were killed and preserved for histological study, four with and three without visible cataracts. Two with the disease were kept alive by transfer to an adequate diet, so that the late stages of the eye changes might be observed. The rest (seventeen) died before showing cataracts, although undoubtedly at least some of them had the disease in an early stage.

Cataracts occurred with a similar frequency in larvae fed the casein diet made with a low fat content by use of skim milk powder. The only reaction to later increase in supply of fat and vitamins was a slight and transient acceleration of growth. Addition of autoclaved yeast to the diet of nine

larvae, to give them six times the original yeast supplement, did not relieve their general debility as noted by Day, Langston and O'Brien ('31) in rats when vitamin G was increased in the cataract producing diet of Sherman and Spohn. Four cases of cataract appeared (66%) among six salamander larvae living when the first case was seen, the last case appearing 7 weeks after the yeast was increased. The other two members of the group died before showing the disease. Four cases of cataract (40%) also appeared among ten larvae given the low fat ration with its vitamin A in the form of carotene doubled after 7 weeks.

*The cataracts.* An early stage of the cataracts was seen rarely (fig. 2) as striae between the equator of the lens and its anterior pole. Usually, the disease did not become evident until the swollen and whitened lens protruded into the anterior chamber of the eye (fig. 3). Dissection showed that the disease was confined to the cortical layers of the lens, and this was confirmed by histological examination.<sup>4</sup> The rate of progress of the disease varied in different animals and bilaterally in the same animal. The gradual decrease of the whiteness of the pupils of the eyes as seen by unaided vision was found to be due to disappearance of the shrinking lens (fig. 4). There was seen one case of bilateral subluxation (figs. 5, 6) which allowed direct observation of the lenses throughout the late stages of the disease.

*Absence of cataracts in larvae fed additional cystine.* It was suspected that the cause of the general poor health and of the cataracts in the salamander larvae lay in the purified casein, chief ingredient of the faulty ration. As the casein was not toxic to rats, its fault was assumed to lie either in its composition or in its utilization by the larval salamanders. Nine larvae fed the low fat ration were also given a low intake of protein (2.4% of the purified casein; total protein 16%) with the carbohydrate increased by dextrin to 78%. No growth occurred in these animals and hence no faulty lens protein was formed; neither did any deaths occur. An

\*The histological conditions will be discussed elsewhere.

edema appearing in the sixth week was relieved by increase of the dietary fat to the usual level, but no other response was evident. Two weeks later, the purified casein was increased to give three protein levels of 30, 55, and 80% each for three larvae. All of the animals began to grow, with the greatest acceleration in those given the middle level. The low content of cystine in casein has long been known to be of biological significance to rats. Accordingly, cystine was added to the diet in the amount which would be present if the dietary protein were beef muscle (Sherman, '37). When the supplement of cystine was started, 2 weeks after growth had been initiated by the protein increase, one case of cataracts was developing in the group. No other case appeared, although these larvae lived longer than those which were fed the higher level of purified casein from the beginning and which suffered early from its inadequacies.

*Repetition with a different strain of A. tigrinum.* Although the cataracts seen in the casein-fed salamander larvae were quite clearly a result of diet, there was a possible relation to strain of the animals. To eliminate this the above work was repeated with two sets of larvae of the same species raised from Wisconsin eggs ('34, '35). In both sets, the diet of purified casein and powdered milk produced cataracts like those previously seen, and no cataracts developed in larvae fed this same diet supplemented with cystine. Other diet groups free from cataracts included seventy-two larvae fed the synthetic diet of beef muscle with different combinations of vitamin D, calcium, and phosphorus (Patch, '35), six larvae given unrefined casein with the milk powder, fifty-four given the diet of purified casein with six different commercial liver extracts (Patch, '36), ten fed raw beef muscle, and fourteen fed raw beef liver.

#### C. CATARACTS PRODUCED BY DIETS OF THE SEPARATE MUSCLE PROTEINS

The results of the above experiments indicated that the cataracts produced in the salamander larvae by the diet of purified casein and powdered milk were due to qualitative

inadequacy of the dietary protein. This was apparently true also of the anemia. Neither of these diseases had appeared in the species when fed the beef muscle ration. However, beef muscle contains several proteins, some fat and minerals, as well as vitamins of the B complex group. In order to use isolated proteins, yet some which were more closely allied in composition to those of the natural diet of the carnivorous salamanders than was the casein of the early experiments, muscle was fractionated and separate diets were made of its most abundant proteins. Quickly-ground cheek muscle from newly-killed cows was extracted with an alkaline-buffered KCl solution at a temperature just above freezing, and the dissolved proteins were precipitated separately by graded dialysis against buffers of progressively lower pH (Weber and Meyer, '33). The precipitated myosin, and then the globulin x in turn, were washed with the precipitating buffer solution to remove traces of the proteins soluble at that pH. Each of these proteins, as well as the extraction residue (chiefly stroma protein), was first washed with distilled water to remove inorganic ions and then was dried and pulverized. The third protein precipitated, namely water-soluble myogen, is accompanied in red muscle by an approximately equal amount of hemoglobin of muscle and of blood not separable from the myogen. To prepare this muscle protein in purer form, the suggestion of Weber was followed and extraction made of the hemoglobin-low white muscle of chicken.<sup>5</sup> The precipitated myogen (Weber, '25) was dialyzed against water to remove inorganic ions, and then pulverized. The three powdered proteins from beef muscle and the four prepared from white muscle of chicken were used as the proteins of seven different rations (table 2), which were fed to *Ambystoma*

<sup>5</sup> The amounts of the muscle proteins obtained from this preparation may serve to illustrate the relative quantities of the four proteins regained and the food quantity required for a set of experimental diets for the salamander larvae. From the white muscle of two chickens, totalling 8 pounds in weight, there were obtained in pulverized form 8.67 gm. of myosin, 6.09 gm. of globulin x, 6.65 gm. of myogen, and 9.77 gm. of the extraction residue.

tigrinum larvae raised from eggs of Tennessee ('36, '37, '38)<sup>6</sup> and of Wisconsin ('39, '40).

*Cataract production.* Comparison was first made of the nutritive value of the extraction residue and the myosin prepared from beef muscle. The larvae fed the extraction residue were small, but well-proportioned and active; they were not anemic and half of the group metamorphosed to the adult state. In contrast to animals fed whole muscle, however,

TABLE 2  
*Composition of the diet for protein variation.*

INGREDIENTS	PROTEIN	CARBO-HYDRATE	FAT	MINERALS	VITAMINS	TOTAL
Protein	60	..	..	..	..	%
Cornstarch	..	27	..	..	..	27
Oil (Wesson)	..	..	10	..	..	10
Minerals <sup>1</sup>	..	..	..	3	..	3
Vitamins <sup>2</sup>					ABDG	
Total	60	27	100	3		100

The non-varying ingredients were mixed in quantity sufficient for 100 gm. of ration, and the mixture was kept frozen in 10 gm. lots until time for use. Before adding to the other ingredients of this mixture, the cornstarch was cooked (Hubbell and Mendel, '27) with a minimum of distilled water to make a paste. In making a ration, the proper amount of the paste mixture was added to the protein and other variants. Distilled water was added to obtain the suitable consistency for feeding.

<sup>1</sup> Osborne and Mendel ('18).

<sup>2</sup> In early work, vitamins were supplied by cod liver oil (2%) and powdered bakers' yeast (1%). Later, AbdoL was used, a capsule supplying 100 gm. of ration with 6200 U.S.P. XI units of vitamin A, 900 of the same units of vitamin D, 45 Sherman units of vitamin B<sub>1</sub> and 10 Sherman units of vitamin G(B<sub>2</sub>).

cataracts developed in four of six living for 2 months or more on the muscle fraction. The other members of the group died without showing the eye disease. The cataracts produced were like those caused by the diet of purified casein and powdered milk, but here the disease was clearly revealed to be independent and primary. In contrast to the good health

<sup>6</sup> The Tennessee eggs were supplied by Dr. R. C. Hutchinson of The Morris Biological Farm of The Wistar Institute.

of the larvae fed the residue diet, those fed the myosin were severely anemic and began to die in the fifth week before any cataracts had appeared. Five remaining animals were fed extraction residue together with the myosin to approximate the relative proportions of these two proteins in whole muscle (Weber and Meyer, '33). Three developed to metamorphosis which was successful in two. Two cases of cataract appeared, one in a metamorphosing animal. The relation of the myosin to the cataracts was not known until larvae of another experimental lot were maintained longer on a diet with myosin as the only protein, and cataracts appeared in one of two surviving the longest. Cataracts were also produced in this second set of animals by a diet of beef globulin x, which was poor for growth and development, but did not cause a marked anemia as the myosin did. Production of cataracts by diets of all four proteins prepared from the white muscle of chicken completed the evidence that one or more substances needed for formation of normal lens protein had been removed from the whole muscle during the extraction process and had not been recovered with any of the separated proteins.

*Control diets not associated with cataracts.* Other larvae of the same origin as those fed the separate muscle proteins were fed artificial diets of powdered whole muscle of beef that had not been treated and diets of powdered muscle that had been denatured first by cooking or by spoiling after alternate freezing and thawing. No cataracts developed in these larvae, in others fed raw beef liver, or those given artificial diets containing liver, halibut muscle, red or white muscle of chicken.

*Preventive experiments.* The cataracts caused by the diets of the muscle proteins exhibited in their development the same changes as the cataracts due to the purified casein diet. Visibility of an early stage was rare, so that curative experiments were not possible. Prevention was not attained by dietary supplementation with riboflavin (1.25  $\mu$ g. per gram of ration), the factor which Day, Darby and Cosgrove ('38)

found effective in preventing cataracts in rats fed the casein and cornstarch diet with low vitamin G content. For these reasons, and because both the casein purification and the muscle extraction had included treatment with dilute alkali, it was thought that there might be a unity of cause for the production of the eye disease by the two sets of diets. Possibly, cystine had been removed during the alkaline-buffered extraction of the muscle proteins as during the treatment of casein by dilute alkali (Jones and Gersdorff, '34). Accordingly, cystine supplementation was tried with the muscle-protein diets. Prevention was not attained when the amount of this amino acid (1.25% of the dietary protein) which had been effective with the diet of purified casein and powdered milk was added to the diet of the residue from the beef extraction. Three cases of cataracts developed among six survivors, while three cases also appeared in a group of five larvae living on the diet without cystine. No cataracts appeared in a second group of five larvae given this diet with twice the earlier supplement. Also, none appeared in a group fed beef globulin X with this amount of the supplement. However, when this supplementation was repeated with larvae of another experimental lot and with muscle proteins of another extraction, complete prevention was not obtained with either the diet of the extraction residue or that containing myosin. Two cases of lens disease developed among six animals surviving longest on the former diet with cystine and one case among three fed the supplemented myosin. Possibly these animals needed a greater supply of cystine. In a group of five larvae of the same lot fed the myosin diet without the cystine, two cases of cataract appeared, one showing unilateral subluxation. The number of cataracts in larvae fed the seven muscle-protein diets, with and without the experimental supplements, was doubtless greater than the total seen (twenty-eight in fifty-five larvae or 51%), because some of the animals that died probably had the disease in an early non-visible stage.

#### D. CATARACT PRODUCTION BY DIETS OF HIGH HEMOGLOBIN CONTENT

At the same time the diet experiments with the muscle proteins were started, groups of the flesh-eating salamander larvae were also placed on different diets of powdered beef-blood preparations whose nutritive values were not known because they had been found distasteful to rats (Mitchell and Hamilton, '29). The rations (table 2) were similar to those of the muscle proteins, with protein supplied by whole blood, defibrinated blood (beef and also turtle), hemoglobin (Bradley and Sansum, '14), or fibrin. The larvae fed these diets grew slowly and did not progress far toward metamorphosis, but they clung to life tenaciously.

*Cataracts of type two.* Diets of the first three blood preparations produced cortical cataracts, but these were of a type very different from those caused by the diets previously described. All of the larvae (100%) fed the diets of high hemoglobin content showed a zoning of the lenses, sometimes evident after a month on the rations. A line of demarcation (fig. 7) inside a clear zone at the lens surface appeared to grow darker and broader. Sometimes there were two or three of these lines parallel to the lens surface, the outer ones appearing fainter. After an extended stationary period, the banding disappeared in 37% of the larvae and was replaced by whitening of the lens cortex (fig. 8). With the whitening, occurring 2 to 6 months after the diet was started, there was a large amount of gelatinous fluid within the lens capsule. This was followed by another period of slow change, during which the cortex grayed gradually, but lost little in size; the lens position was not changed, and the subcapsular fluid was retained. The bilateral difference in rate of progress of the disease was less than that which occurred with cataracts of the first type. The histological changes of the two types of cataract were strikingly different.<sup>7</sup>

The lens zoning did not appear in the larvae fed blood fibrin, nor in those fed a diet of beef muscle one-fourth replaced by

<sup>7</sup> See footnote 4, page 368.

heme (Anson and Mirsky, '30) and thus containing more than three times the amount of that pigment in the ration of hemoglobin. The line did not appear in the small larvae fed a similar diet of heme and myosin; but cataracts typical of a myosin diet developed in the larva living longest. If the cataracts produced by the blood diets had been due to the heme pigment, the presence of more than the natural amount of heme should have brought cataracts of the second type, regardless of the attending protein. A faint line of demarcation appeared near the lens surface in the larvae maintained without growth by a diet of the inseparable mixture of the hemoglobins of muscle and blood with the water-soluble myogen extracted from beef muscle. The same faint zoning appeared in eyes of the larvae losing size on a diet of separated denatured globin and heme in the natural proportion, and in similar larvae fed a diet of the globin without heme. These larvae survived to the point of development of mature cataracts only if transferred to the more nutritious diet of dried blood.

*Effect of diet change in suppressing cataract development.* Because the second type of cataract always had a visible early stage, attempts to cure the disease or to halt its progress were feasible. When larvae with a definite lens zoning were placed on a diet adequate for lens health, such as raw muscle or raw liver, the line in the lens did not disappear, but became buried by a band of uninterrupted transparency (fig. 9) which halted progress of the diseased condition. The width of the newly-formed zone of normal tissue was related to the growth value of the new diet, and was greatest with liver feeding.

*Preventive factors.* Lens zoning and the subsequent development of cataracts in larvae fed the rations of high hemoglobin content were not prevented by a supplement of riboflavin (1  $\mu$ g. per gram of ration) nor by methionine (2.5% of the total protein). High supplementation with cystine (7% of the total protein or 5% of the total solids of the ration) was ineffective. In contrast to casein, globin is poorly supplied.

not only with cystine, but also with the other amino acids present in glutathione. This tripeptide, long recognized to be important in lens metabolism (Krause, '34), has been found to be most abundant where new lens fibers are forming. When a group of larvae was fed the diet of whole blood supplemented with glutamic acid (15% of the protein) and cystine (3.3%), a definite lens zoning had appeared in only one-fourth of the animals at the end of a month and this proportion was held through 6 months with no further development of the disease. In larvae given the blood diet with glutamic acid and cystine, plus glycine (3%) as well, the zoning was further retarded and vague. The addition of arginine (4%), which is present in globin in small amount (Bergman and Niemann, '37), with the other three amino acids did not enhance their effect. That the action of the three acids was partly due to cystine was proved by an earlier and more definite lens zoning in larvae receiving the blood diet supplemented with glutamic acid and glycine but no cystine. Failure to secure complete prevention of cataracts by a liberal supply of the three acids suggested an inadequacy of these acids in the uncombined form or an insufficiency of some other dietary component. Development of the second type of lens disorder, rather than that produced by the casein diet (containing abundant glutamic acid and a fair amount of glycine) with no cystine supplement, gave evidence that an undetermined irregularity of the diet had been directing the specific nature of the lens changes.

#### DISCUSSION

Need for a cystine supplement with the diet of purified casein and not with that of unrefined casein was explained by the data of Jones and Gersdorff ('34) who found that repeated treatment of casein by means of dilute alkali not only removes the cystine adhering to the casein in its separation from the lactalbumin of milk, but also causes a disintegration of the cystine inherent in the casein molecule. In the present experiments the cystine supplement acted specifically on the formation of lens protein and did not hasten growth or prevent

the anemia. Other evidences of a specific relation of this amino acid abundant in ectoderm to the health of the ectodermally-derived lens have been reported in work with other animal forms. When Day, Langston and Cosgrove ('34) replaced the casein of the Sherman and Spohn diet deficient in vitamin G by the higher cystine-containing egg albumin, no cataracts appeared in their experimental rats although a marked dermatitis developed. These workers also found that chickens fed a diet with egg albumin as the source of protein had severe dermal lesions and only slight eye changes, while chickens given the same diet with casein showed cataracts and only a mild dermatitis. Bourne and Pyke ('35) reported an absence of cataracts in rats fed the regular diet of Sherman and Spohn when it was supplemented by cystine (1%). Bourne and Young ('34) expressed the opinion that the cause of naphthalene cataracts in rabbits might be due to the fact that these animals detoxicate this aromatic hydrocarbon by a conjugation with cysteine, thus depriving the lens of that amino acid. Yudkin and Geer ('40) reported prevention of cataracts due to high galactose rations (Mitchell, '35), when a high supplement (4%) of cystine was used. In the ration of purified casein and powdered milk, which produced cataracts in the salamander larvae, the galactose content was low (7%). Moreover, the same galactose percentage was present in the diet when the preventative cystine was added and the same amount was present with the preventive diet of commercial casein. No galactose was included in the diets of the separate muscle proteins nor in the diets of high hemoglobin content; all of these diets produced cataracts.

The primary nature of the lens disease, shown by the specific action of cystine as a supplement to the diet of purified casein, was emphasized by the occurrence of cataracts in animals developing normally through metamorphosis on the diets containing the stroma protein from beef muscle. The preventive action of cystine supplementation with these diets with some groups of larvae and not with others suggested different margins between the cystine need and the supply. More

cystine may have been lost in the later muscle extractions, or the animals fed these materials may have had less cystine in their preliminary feeding. Possibly some additional substance needed for lens health was lost during the later extractions. Curtis, Hauge and Kraybill ('32) found that "a white opaqueness of the eye . . . quite different from ophthalmia due to vitamin deficiency" appeared in rats fed a diet with inadequate protein when it was supplemented by cystine plus tyrosine, but did not appear when the diet was supplemented by cystine plus tryptophane. Recently, Totter and Day ('41) have produced cataracts in rats fed a diet deficient in tryptophane but supplemented with cystine. The effect of this diet supplemented with tryptophane and not with cystine has not been shown. Few data have been published on the amino acid content of the muscle proteins. According to Bailey ('37), myosin does not contain a significantly small amount of tryptophane, but its content of cystine is low.

Studies of the second type of cataract produced in the salamander larvae offer definite returns because of the ease and regularity with which the early stage can be obtained and because of the slow progress of the disease. Curative attempts may be made as well as an experimental analysis of suppressing and preventive factors. The relation between this type of the lens disease and the general health of the animals has yet to be determined.

The lens is a structure peculiarly susceptible to dietary influence because of its continued growth throughout life (Bourne, '37). For this reason, the experimental production of cataracts in growing animals and the development of cataracts in adults are not without some similarities. Moreover, the fundamental metabolic processes of the lower and higher animal forms are the same. The small carnivorous salamander larvae with their low total food intake and their high protein requirements are especially suitable for the study of problems of protein metabolism, as in the changing lens.

## SUMMARY

Two types of cortical cataract have been produced in larvae of the tiger salamander, *Ambystoma tigrinum*, by three series of synthetic diets differing in their chief constituent, protein. Eyes were normal in larvae fed raw muscle or liver or artificial diets containing these tissues as chief ingredients.

Cataracts rarely visible in an early stage as radiating striae, with swelling followed by extensive shrinking, were produced in 43% of the animals on diets of purified casein and powdered milk and in 51% on diets containing separate muscle proteins. Preparation of each of these materials involved the use of dilute alkali, known to disintegrate cystine. The quantities of this amino acid used as supplements resulted in prevention of the lens disease in larvae fed the casein diet and in two groups of those fed the muscle-protein diets. Riboflavin was not a preventative.

Cataracts with an always visible early line of zoning parallel and near to the lens surface appeared in larvae fed high hemoglobin rations. The stage of whitening, which occurred in 37% of the animals, was accompanied by a large fluid content within the lens capsule and subsequent changes were slow. Prevention was not attained with riboflavin nor with a high supplement of cystine alone. A combination of the three amino acids of glutathione reduced the early symptoms of the disease and halted its development, but did not bring about complete prevention.

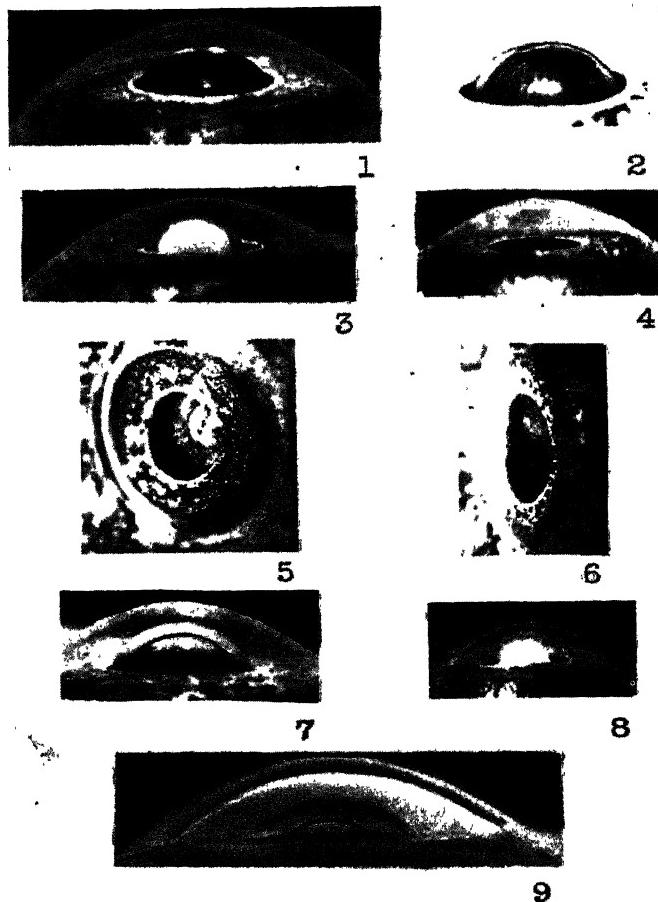
## ACKNOWLEDGMENT

The chemicals and equipment for making the muscle extractions were supplied by the Department of Physiological Chemistry of the University of Wisconsin Medical School. Progress of the work was aided by the interest and encouragement of Prof. C. A. Elvehjem of the Department of Biochemistry, School of Agriculture, University of Wisconsin.

## LITERATURE CITED

- ANSON, M. L., AND A. E. MIRSKY 1930 Protein coagulation and its reversal. The preparation of insoluble globin, soluble globin, and heme. *J. Gen. Physiol.*, vol. 13, pp. 469-476.
- BAILEY, K. 1937 Composition of the myosins and myogen of skeletal muscle. *Biochem. J.*, vol. 31, pp. 1406-1413.
- BERGMAN, M., AND C. NIEMANN 1937 On the structure of proteins: Cattle hemoglobin, egg albumin, cattle fibrin, and gelatin. *J. Biol. Chem.*, vol. 118, pp. 301-314.
- BOURNE, M. C. 1937 Metabolic factors in cataract production. *Physiol Rev.*, vol. 17, pp. 1-27.
- BOURNE, M. C., AND L. YOUNG 1934 The metabolism of naphthalene in rabbits. *Biochem. J.*, vol. 28, pp. 803-808.
- BOURNE, M. C., AND M. A. PYKE 1935 The occurrence of cataract in rats fed on diets deficient in vitamin B<sub>2</sub>. *Biochem. J.*, vol. 29, pp. 1865-1871.
- BRADLEY, H. C., AND W. D. SANSUM 1914 Some anaphylactic reactions. I. Hemoglobin specificity. *J. Biol. Chem.*, vol. 18, pp. 497-506.
- CURTIS, P. B., S. M. HAUGE AND H. R. KRAYBILL 1932 The nutritive value of certain animal protein concentrates. *J. Nutrition*, vol. 5, pp. 503-517.
- DAY, P. L., W. C. LANGSTON AND C. S. O'BRIEN 1931 Cataract and other ocular changes in vitamin G deficiency. An experimental study on albino rats. *Am. J. Ophth.*, vol. 14, pp. 1005-1009.
- DAY, P. L., W. C. LANGSTON AND K. S. COSGROVE 1934 The appearance of cataract and dermatitis in experimental animals given vitamin G deficient diets containing casein and egg albumin. *J. Nutrition*, vol. 7, p. 12 (proc.).
- DAY, P. L., W. J. DARBY AND K. S. COSGROVE 1938 The arrest of nutritional cataract by the use of riboflavin. *J. Nutrition*, vol. 15, pp. 83-90.
- HUBBELL, R. B., AND L. B. MENDEL 1927 Zinc and normal nutrition. *J. Biol. Chem.*, vol. 75, pp. 567-586.
- JONES, D. B., AND C. E. F. GERSDORFF 1934 The effect of dilute alkali on the cystine content of casein. *J. Biol. Chem.*, vol. 104, pp. 99-106.
- KRAUSE, A. C. 1934 The Biochemistry of the Eye. Chapter VIII. The Chemistry of the Lens. Baltimore. The Johns Hopkins Press.
- MITCHELL, H. H., AND T. S. HAMILTON 1929 The Biochemistry of the Amino Acids. The Chemical Catalog Company. New York.
- MITCHELL, H. S. 1935 Cataract in rats fed on galactose. *Proc. Soc. Exp. Biol. and Med.*, vol. 32, pp. 971-973.
- OSBORNE, T. B., AND L. B. MENDEL 1918 The inorganic elements in nutrition. *J. Biol. Chem.*, vol. 34, pp. 131-139.
- PATCH, E. M. 1934 Cataract as a result of dietary deficiency in larval *Ambystoma tigrinum*. *Science*, vol. 79, pp. 57-58.
- 1935 Calcifying factors in the diet of salamander larvae. *Science*, vol. 81, pp. 494-495.
- 1936 Dietary production and prevention of anemia in larval *Ambystoma*. *Science*, vol. 83, pp. 560-561.
- 1941 Cataracts in *Ambystoma tigrinum* larvae fed experimental diets. *Proc. Soc. Exp. Biol. and Med.*, vol. 46, pp. 205-207.

- PATCH, E. M. Laboratory controlled development and growth of urodeles. (In press.)
- SHERMAN, H. C. 1937 Chemistry of Food and Nutrition. Fifth Edition. New York, Macmillan Co.
- SPRINGER, A. 1909 A study of growth in the salamander. *Diemyctylus viridescens*. *J. Exp. Zool.*, vol. 6, pp. 1-68.
- TODD, W. R., C. A. ELVEHJEM AND E. B. HART 1934 Zinc in the nutrition of the rat. *Am. J. Physiol.*, vol. 107, pp. 146-156.
- TOTTER, J. R., AND R. L. DAY 1941 Cataract and other ocular changes resulting from tryptophane deficiency. *Proc. Am. Soc. Biol.*, *J. Biol. Chem.*, vol. 140, pp. cxxxiv-cxxxv.
- WEBER, H. H. 1925 Das Kolloidale Verhalten der Muskeleiweiszkörper. I. Mitteilung: Isoelektrischer Punkt und Stabilitätsbedingungen des Myogens. *Biochem. Zeitschr.*, Bd. 158, S. 443-472.
- WEBER, H. H., AND K. MEYER 1933 Das Kolloidale Verhalten der Muskeleiweiszkörper. V. Mitteilung: Das Mengenverhältnis der Muskeleiweiszkörper in seiner Bedeutung für die Struktur des quergestreiften Kaninchenmuskels. *Biochem. Zeitschr.*, Bd. 266, S. 137-152.
- YUDKIN, A. M., AND H. A. GEER 1940 An investigation of experimental cataracts in the albino rat: Clinical implications. *Arch. Ophth.*, vol. 23, pp. 28-40.



- 1 Normal lens by reflected light.  $\times 10$ .
- 2 Early stage of cataract caused by diet of purified casein. Striae between equator and anterior pole of the lens.  $\times 20$ .
- 3 Stage of maximum swelling.  $\times 10$ .
- 4 Depressed pupil after shrinking of lens.  $\times 10$ .
- 5 Stage of swelling in case of subluxation.  $\times 10$ .
- 6 Subluxated lens, 39 days later, during shrinking process.  $\times 10$ .
- 7 Dark line between transparent outer and inner layers of normal lens tissue.  $\times 20$ .
- 8 Stage of swelling in cataract of type II.  $\times 10$ .
- 9 Wide outer band of normal lens tissue formed after transfer of animal to diet of raw liver.  $\times 20$ .

The photographs were made with equipment of the Department of Anatomy, University of Wisconsin Medical School, under the direction and with the help of Prof. H. W. Mossman.

# THE INFLUENCE OF PREVIOUS REGIMES OF PROTEIN FEEDING ON THE ENDOGENOUS NITROGEN METABOLISM OF RATS

R. B. FRENCH, J. I. ROUTH AND H. A. MATTILL

*Biochemical Laboratory, State University of Iowa, Iowa City*

ONE FIGURE

(Received for publication June 16, 1941)

In the determination of biological value of proteins by the method of nitrogen balance the most important question concerns the length of time the animals must be kept on a nitrogen-free diet to assure that the nitrogen excretion may reasonably be called endogenous only. Since the early work of Voit (1866) it has been axiomatic that the higher the level of protein on which an animal is living, the longer the time required to rid the organism of "circulating" protein. To secure uniformity of results Hindhede ('26) rightly insisted upon the necessity of an adequate preliminary period. Under ordinary conditions in rats this interval has been accepted as 1 to 4 days. Mitchell and his co-workers ('22, '23-'24) found that a rapid excretion of nitrogen occurred during the first 2 days of a protein-free diet, after which in some cases, an approximate constancy of excretion obtained; these results were confirmed by others (Mason and Palmer, '35; Chick et al., '35) on adult male rats. On the other hand, Ashworth and Brody ('33) found that with growing rats constancy might not be attained in less than 3 to 4 weeks. When a diet containing 4% of egg protein is used in place of the nitrogen-free diet as a conditioning ration (Mitchell and Carman, '26), the deprivation of protein is less, and appetite and body weight should be better maintained, but any periods of realimentation involve protein storage

since the experimental diets are of necessity always low in protein. The status of this and of other aspects of the subject was reviewed by Boas-Fixsen ('35).

The question of the true endogenous level became of particular importance in connection with a report of the biological value of bread proteins (French and Mattill, '35) which contained data and conclusions from experiments on human subjects and on young and adult rats. The figures from adolescent rats were considered unsatisfactory because nitrogen excretion on the nitrogen-free diet failed to become stationary even after 10 days. It seemed possible that in such animals an exceptional amount of circulating protein might be a normal accompaniment of physiological development. This appears to be only partially true; the conditions responsible for the abnormally high protein reserve were again applied, this time purposely, and the same results were obtained, all of which are here briefly described.

In the original study the biological values were measured on the same lot of about thirty rats during the three stages of growth mentioned. In the experimental periods the animals were on protein-free diets and on bread diets containing 5% of protein. For realimentation between these periods they were given a complete diet, *ad libitum*, until they reached the next stage. The clue to what had happened appeared when the observations on maturing animals were repeated several years later, some of the animals having gone through a similar balance study during adolescence, others being taken directly from stock.

#### EXPERIMENTAL

The percentage composition of the nitrogen-free diet was as follows: corn starch 77, salts 4, cellu flour 2, hydrogenated vegetable oil 15, cod liver oil 2. This diet contained 0.05% nitrogen. One vitamin<sup>1</sup> tablet or its equivalent was fed daily; it supplied 5-7 mg. of nitrogen. The animals were kept in wire cages,  $\frac{1}{2}$  inch mesh, and the urine was absorbed on nitrogen-free filter paper prepared by moistening with an

<sup>1</sup> Harris, from the Harris Laboratories, Tuckahoe, New York.

alcoholic solution of boric acid and drying. At the end of each 2-day period the feces were removed for analysis and the paper was reduced to pulp by mechanical stirring in dilute acid solution, filtered on a Buchner funnel and filtrate and washings made to volume from which aliquots were taken for analysis (Kjeldahl).

The average course of urinary nitrogen excretion of all the animals (in milligrams per 100 gm. body weight) is given in the chart. A preceding low protein diet showed its effect in one group of the young animals (C). The addition of milk and lettuce to the dog-biscuit stock diet (A) also showed its effect, since even after 12 days the nitrogen output had not become constant; in animals (B) taken directly from stock diet urinary nitrogen became relatively constant by the eighth day. The differences between these three groups are such as would be expected.

Of the adolescent animals, those coming directly from stock diet and having no previous experimental history (E) had a relatively constant nitrogen output after the fourth day. Surprisingly, those (D) which had undergone realimentation for 3 weeks immediately succeeding a 3-week period on non-protein and low protein diets had not arrived at a constant output even after 10 days. Unfortunately the analytical data were not available soon enough to demonstrate the necessity of prolonging the non-protein feeding period.

In contrast, the grown animals came to relatively constant excretion in 4 to 6 days, irrespective of whether they had 2 months previously suffered from brief protein starvation (H) or were taken directly from stock diet (G). The group of ten slightly smaller and younger animals (F) (a part of lot E) had been on ad libitum stock diet for 2 weeks following a period of 18 days on nitrogen-free diet. At the end of 4 days their nitrogen excretion had fallen to a relatively low level, but this rose again and did not decline to the 4-day level of groups G and H until after 16 days. Figures for fecal nitrogen obtained throughout these studies parallel those for urinary nitrogen, but with much smaller fluctuations.

The interpretation of the behavior of the young rats is obvious; their endogenous nitrogen was related to the previous dietary nitrogen. The adolescent animals (D) had been deprived of protein during a period of rapid development. In the 3 weeks of ad libitum feeding they more than compensated for the loss and had stored an excess of protein much above that which they would normally have accumulated (E). How long a period would have been required for a reduction of this excess to normal levels is uncertain. In adult animals (H), a 2 months period following brief protein deprivation was sufficient to permit this excess to be lost. Furthermore their demands for protein were largely for maintenance and less

#### EXPLANATION OF CHART

*Excretion of urinary nitrogen (mg. per 100 gm. body weight) by rats on N-poor diet following diets with various levels of nitrogen intake.*

GROUP	PREVIOUS DIETARY HISTORY	NUM- BER OF ANIMALS	BODY WEIGHTS DURING N-FREE PERIOD		LENGTH OF PERIOD	FRACTION OF ORIGINAL WEIGHT LOST
			Beginning	End		
A	Dog biscuit, milk, lettuce	10 ♀	gm. 58 (45-74)	gm. 42	days 12	% 28
B	Dog biscuit	8 ♂, 8 ♀	50 (43-64)	36	18	28
C	3 weeks on low-protein	31 ♀	62 (45-80)	50	10	20
D	Dog biscuit for 3 weeks, following 20 days of non-protein (10) and low protein (10) diet	31 ♀	136 (90-158)	97	10	29
E	Dog biscuit	7 ♂, 12 ♀	126 (95-147)	99	18	21
F	Dog biscuit for 2 weeks following 18 days on N-free diet	6 ♂, 4 ♀	181 (170-211)	131	18	28
G	Dog biscuit	6 ♀	215 (202-224)	174	18	19
H	Dog biscuit for 2 months following several peri- ods of low- and non- protein diets	31 ♀	184 (151-211)	169	12	8

for growth, the urge was less and the smaller shortage was restored more quickly. Losses in body weight in the nitrogen-free period were greater when realimentation immediately preceded this period (compare D and F with E and G); they were also greater in young animals (A, B) than in older animals (G, H).

The contrast in behavior between groups F and G is particularly disturbing because many studies of biological value have been made on animals within this weight range. The previous deprivation of protein suffered by the animals in group

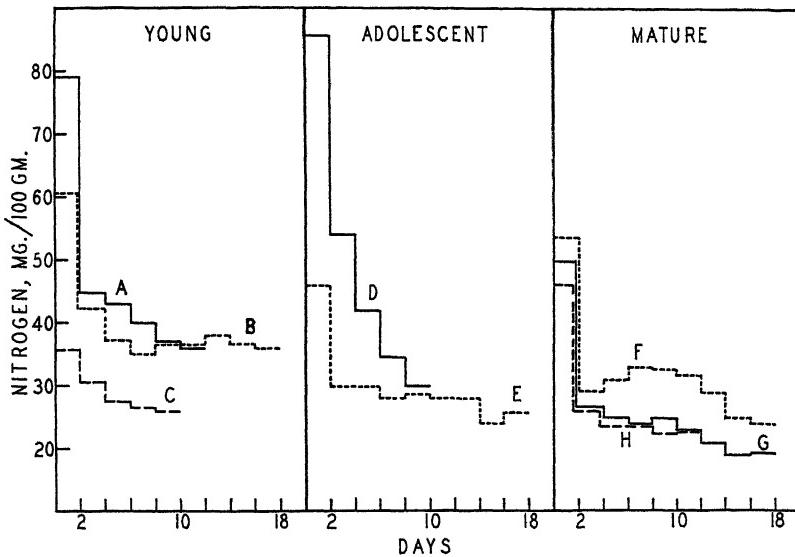


Chart 1

F may well explain their higher nitrogen output after 2 weeks of ad libitum feeding, as compared with the nitrogen excretion of the G animals, but it does not explain their subsequent increased and continued higher level of nitrogen output, while that of the G animals again began to decline after 10 days on the nitrogen-free diet. Even after ad libitum stock feeding without any experimentation, endogenous nitrogen excretion sometimes takes this course (Ashworth, '35) in maturing but

not in young animals. Obviously, the use of short non-protein feeding periods would lead to quite different figures for the biological value of proteins fed to animals like those in the F and G groups.

Many of the studies on biological value may have been complicated by this unforeseen departure from the ordinary course of endogenous nitrogen excretion.

#### SUMMARY

When rats employed in the determination of biological value of proteins by the balance method are subjected to repeated low protein or non-protein regimes interspersed with periods of realimentation they overcompensate for the loss of body protein in these periods, and accumulate unusual stores. As compared with controls such animals require a longer time on a low protein diet before their urinary nitrogen falls to an acceptable endogenous level and also suffer a greater loss in weight. This was particularly noted in maturing animals (100-200 gm. in weight) which are commonly used for such studies and the significance of these observations is briefly discussed.

#### LITERATURE CITED

- ASHWORTH, U. S. 1935 Endogenous nitrogen and basal energy relationships during growth. *Missouri Agr. Exp. Sta. Res. Bull.* 223.
- ASHWORTH, U. S., AND S. BRODY 1933 Growth and development with special reference to domestic animals. XXVII. Endogenous urinary nitrogen and total creatinine excretion in rats as functions of dietary protein level, time on nitrogen-free diets, age, body weight, and basal metabolism. *Ibid.*, 189.
- 1933 XXVIII. Decline of endogenous nitrogen excretion per unit weight with increasing weight in growing rats, and its relation to the decline in basal metabolism. Decline in live weight, nitrogen and energy metabolism with the advance of the period of nitrogen starvation and the influence of live weight and of preceding level of protein intake on these declines and on survival periods. *Ibid.*, 190.
- BOAS-FIXSEN, M. A. 1935 The biological value of protein in nutrition. *Nutrition Abstracts and Reviews*, vol. 4, p. 447.
- CHICK, H., J. C. D. HUTCHINSON AND H. M. JACKSON 1935 The biological value of proteins. VI. Further investigation of the balance sheet method. *Biochem. J.*, vol. 29, p. 1702.

- FRENCH, R. B., AND H. A. MATTILL 1935 The biological value of the proteins of white, wheat and rye breads. *Cereal Chemistry*, vol. 12, p. 365.
- HINDHEDE, M. 1926 The biological value of bread protein. *Biochem. J.*, vol. 20, p. 330.
- MASON, I. D., AND L. S. PALMER 1935 Utilization of gelatin, casein and zein by adult rats. *J. Nutrition*, vol. 9, p. 489.
- MITCHELL, H. H. 1923-1924 A method of determining the biological value of protein. *J. Biol. Chem.*, vol. 58, p. 873.
- MITCHELL, H. H., W. B. NEVENS AND F. E. KENDALL 1922 The relation between the endogenous catabolism and the non-protein constituents of the tissues. *J. Biol. Chem.*, vol. 52, p. 417.
- MITCHELL, H. H., AND G. G. CARMAN 1926 The biological value of the nitrogen of mixtures of patent white flour and animal foods. *J. Biol. Chem.*, vol. 68, p. 183.
- VOIT, C. 1866 Ueber die Verschiedenheiten der Eiweisszersetzung beim Hungern. *Z. Biol.*, vol. 2, p. 307.



## OBSERVATIONS ON INDUCED CARIES IN RATS

### III. EFFECT OF FLUORIDE ON RAT CARIES AND ON COMPOSITION OF RATS' TEETH

F. J. McCLURE<sup>1</sup>

*National Institute of Health, United States Public Health Service,  
Bethesda, Maryland*

TWO FIGURES

(Received for publication April 23, 1941)

Epidemiological studies relate a partial prevention of human dental caries to the occurrence of fluorides in drinking water (Dean, '38; Dean, Jay, Arnold, McClure and Elvove, '39; Dean, Jay, Arnold and Elvove, '41 a). Induced dental caries in rats also is inhibited by the ingestion of fluoride (Miller, '38; Finn and Hodge, '38; McClure and Arnold, '41). These effects may be due to: (a) inhibition of bacterial action on food residues (Lundsgaard, '30), or dental tissue (Robison and Rosenheim, '34); (b) a systemic effect of fluoride on tooth formation (Armstrong and Brekhus, '38; Cox, Matuschak, Dixon, Dodds and Walker, '39; Volker, '39; Dean, Jay, Arnold and Elvove, '41 b), or (c) a modified saliva (Cheyne, '40; Wills, '40; McClure, '41).

Enamel may become hypoplastic (mottled) as a result of fluoride ingestion during the period of dental calcification. Fluoride has no known post-eruptive effect on human dental enamel, although certain data (Perry and Armstrong, '41) indicate that in rats the oral enamel surface may adsorb fluorides from drinking water. It remains to be demonstrated that post-eruptive ingestion, or a local oral treatment with fluoride, will add significant quantities of fluorine to human enamel, or by some other action inhibit human dental caries.

<sup>1</sup> From the Division of Infectious Diseases, in cooperation with the Division of Chemistry and the Division of Chemotherapy.

### EXPERIMENTAL

The experimental plan follows a previous study (McClure and Arnold, '41). Groups of rats aged 23-25 days, sixteen to twenty-three litter mates in control and corresponding test groups, were on a test period of 15 weeks. A cornmeal caries-producing diet was fed to all groups, *ad libitum*. The caries effect of this diet is generally attributed to the size of the corn particle (Lilly and Wiley, '34). The diets as used averaged 0.3-0.5 ppm. fluorine. Except in two test groups fluoride was added via drinking water. Water intake was not controlled, the results being based, therefore, on fluoride concentration of water or food, and on fluoride given by subcutaneous injection. Comparisons are made between a test group and its corresponding control (McClure and Arnold, '41).

After examining the molar teeth for occlusal caries (Cox, Dodds, Dixon and Matuschak, '39), pooled samples of the crowns of the molars and the erupted part of the incisors of the various groups were pulverized to pass a 60-mesh sieve, the dentine and enamel separated (Manley and Hodge, '39), and fluorine (McClure, '39 a) and ash determined. Pooled samples of the combined dentine and enamel of carious and non-carious rats' molars were analyzed for ash, calcium, phosphorus, and fluorine. Certain of the data was obtained from other experiments (McClure and Arnold, '41; Arnold and McClure, '41).

### RESULTS

Fluorides equalling 50 and 100 ppm. fluorine in water, and 125 ppm. in food, inhibit rats caries decisively (table 1 and fig. 1). It may be concluded that 10 ppm. fluorine in water also inhibited caries as studied. Thus, in fifty-six possible comparisons between litter mate control and test rats, the 10 ppm. fluorine rat showed a lower caries score in thirty-eight instances. The odds are 1 to 259 against this being a fortuitous happening (Student, '25). Rats ingesting 5 ppm. fluorine in water were not different from their controls. The effect of injected fluorine on induced rat caries appears to be negative,

TABLE 1  
Effect of fluoride on rat caries.

TREATMENT	NUMBER OF RATS PER GROUP	RATS WITH CARIES	CARIOUS MOLAR TEETH	CARIOUS MOLAR TOOTH AREAS	AVERAGE CARIES SCORE <sup>1</sup>	
					All rats	Carious rats
Control A	23	per cent 95.6	per cent 27.5	per cent 20.6	17.1	17.9
10 ppm. F. in water	23	69.6	14.9	10.5	8.2	11.8
Control B	27	100.0	35.2	31.4	27.6	27.6
10 ppm. F. in water	24	79.2	26.7	18.8	15.6	19.7
100 ppm. F. in water	26	57.7	13.8	7.8	6.2	16.1
Control C	26	80.8	24.4	20.6	18.0	22.3
5 ppm. F. in water	25	88.0	24.3	20.0	17.9	20.3
50 ppm. F. in water	25	52.0	10.7	5.5	4.0	7.6
Control D	25	76.0	24.7	15.8	10.6	13.9
125 ppm. F. in food	25	12.0	1.0	0.4	0.2	1.3
Control E	23	100.0	27.9	19.4	15.7	15.7
10 ppm. F. in water	20	80.0	22.1	16.6	14.0	17.4
NaF injected	24	83.0	22.2	18.8	16.2	19.4

<sup>1</sup> There are a total of thirty-four areas distributed over the twelve molar teeth in which areas cavities are most likely to occur. Depending on its size, each cavity is given a score of 1, 2, or 3 (see Cox, Dodds, Dixon and Matuschak, '39).

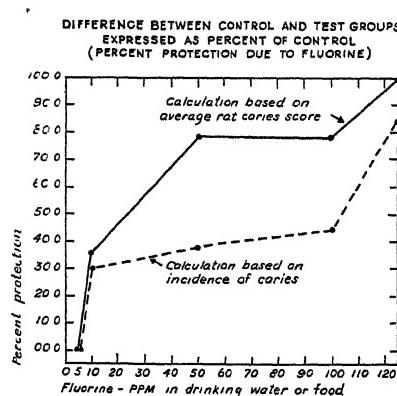


Figure 1

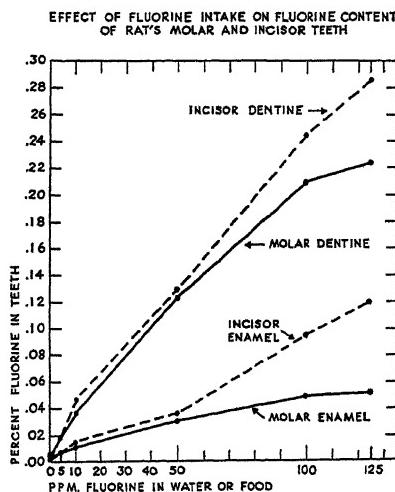


Figure 2

although these rats received an average daily intake of fluorine approximately equal to 50 ppm. of fluorine in drinking water.<sup>2</sup>

The ash of molar and incisor dentine and enamel was not affected by fluoride. Similarly there was no difference in ash, calcium, phosphorus, and fluorine of mixed dentine and enamel of molars of carious rats, as compared with non-carious rats (table 2).<sup>3</sup>

TABLE 2  
*Ash, calcium, phosphorus, and fluorine in molar teeth of carious and non-carious rats.*

TREATMENT	NUMBER OF RATS	AVERAGE CAVITIES SCORE	COMPOSITION OF MIXED DENTINE AND ENAMEL			
			Ash per cent	Ca per cent	P per cent	F per cent
Control A non-carious	5	0.0	78.2	29.1	15.1	.0031
Control A carious	17	17.9	77.0	28.9	14.7	.0035
10 ppm. F. non-carious	10	0.0	77.7	28.9	14.8	.0297
10 ppm. F. carious	13	11.8	75.3	27.7	14.1	.0291
Control B carious	27	27.6	76.9	28.9	14.4	.0082
10 ppm. F. non-carious	6	0.0	78.3	28.7	14.7	.0339
10 ppm. F. carious	18	19.7	77.5	28.5	14.9	.0365
100 ppm. F. non-carious	12	0.0	77.4	29.4	14.5	.1866
100 ppm. F. carious	14	16.1	77.4	29.1	14.8	.2009
Control C non-carious	5	0.0	79.1	28.8	15.4	.0046
Control C carious	21	22.3	77.5	28.5	14.8	.0035
5 ppm. F. non-carious	4	0.0	79.8	29.4	15.2	.0192
5 ppm. F. carious	21	20.3	76.8	28.6	14.5	.0245
50 ppm. F. non-carious	13	0.0	77.4	29.1	14.9	.1140
50 ppm. F. carious	12	7.6	78.3	29.1	14.9	.1069
Control D non-carious	8	0.0	79.2	29.8	14.9	.0025
Control D carious	17	13.9	78.3	29.7	14.6	.0030
125 ppm. F. <sup>1</sup> non-carious	22	0.0	78.9	29.7	15.0	.1842

<sup>1</sup>Fluoride in food.

<sup>2</sup>These injection rats (Arnold and McClure, '41) received 0.5 mg. fluorine as sodium fluoride injected subcutaneously every other day for the first 24 days and every day thereafter for the remaining experimental 75-76 days. At the start of the regular injection period, these rats averaged 41.3 days old, as compared with their controls aged 32.8 days, a delay caused by a local irritation which developed at the site of the first injection. An age factor therefore might have influenced these older rats slightly in caries response. This group of rats, however, did not differ significantly from the controls.

<sup>3</sup>Samples of mixed dentine and enamel, as prepared from carious teeth, may have contained a less than normal quantity of enamel as a result of their carious condition. This would tend to decrease the ash results abnormally on these samples.

## DISCUSSION

The course of prevention by fluoride of induced rat caries (fig. 1) appears to support an anti-enzymatic mode of action. Inhibition occurred at the low level of 10 ppm. of fluorine; it was equally pronounced at 50 ppm. and 100 ppm., and was practically complete at 125 ppm. Since the observed action was not similarly proportional to fluoride deposited in the teeth, there is the suggestion of an effect on reactions occurring locally within the oral cavity. In this connection also, it may be noted that comparison of the two curves (fig. 1) indicates fluoride was perhaps less effective in preventing the initiation of caries (caries incidence) than in retarding its development (caries score).

On the basis of indirect evidence, the etiology of rat caries itself appears to involve an enzyme action within the oral cavity. Thus iodoacetic acid, which, like fluoride, is an active anti-enzymatic substance, inhibits rat caries (Miller, '38; McClure and Arnold, '41). Likewise fluoride which does not reach the oral cavity, i.e., when given by injection, is not caries-inhibitory (Arnold and McClure, '41). In conjunction with the indicated anti-enzymatic effects of fluoride, a bactericidal effect on oral flora is not eliminated.

Results of fluorine analysis of the rats' teeth demonstrate a considerable retention of fluorine by molar dentine and enamel as occurring after tooth eruption (fig. 2). In regard to the rat's incisors, which are continuously erupting, deposition of fluorine is not unusual (McClure, '39 b). However, the similarity of the added quantities of incisor fluoride and molar fluoride<sup>4</sup> particularly for 5, 10, and 50 ppm. rats,

<sup>4</sup>The rat's first and second molar teeth are fully erupted by the twenty-fifth day; the third molars are fully formed at this time, but are not erupted until about the thirtieth day. The apposition of primary dentine in rats' molars (Hoffman and Schour, '40) may possibly account for a part of the fluorine increment in molar dentine. Molar enamel growth, however, is presumably complete at about 10 days of age (Hoffman and Schour, '40). A post-eruptive enlargement of rat molars as reported by Donaldson ('34) was not confirmed by Wood ('35) nor by Hoffman and Schour ('40).

As was to be expected, the rats' incisor teeth showed the effects of fluorosis. The molar teeth were normal in appearance.

implies the acquisition of fluorine by incisors following the developmental stages.

It is particularly significant in these regards that the molar enamel of fluoride injected rats averaged 0.0131% fluorine as compared with 0.0034% fluorine for controls (Arnold and McClure, '41). Since it is doubtful that this increase in enamel fluoride was due to adsorption from saliva (McClure, '41; Willis, '40), these data seemingly indicate that the erupted dentine and enamel retained fluoride from the systemic circulation. Other evidence in the data supports this belief. Thus, the ratio of fluorine in dentine and enamel for molars and incisors is as follows:

Fluoride		10	10	10	50	100	125
treatment (ppm.)							
Molar teeth (ratio)		3.74	3.23	3.23	3.96	4.20	4.31
Incisor teeth (ratio)		2.52	3.43	3.11	3.47	2.59	2.38

This relatively constant proportion of fluorine deposited in these two dental tissues should doubtless be ascribed to a regular systemic retention, which is seemingly unaffected by oral surface adsorption of fluoride.

The property of the oral enamel surface as an adsorbent of water-borne fluoride has been tested recently by Perry and Armstrong ('41). Their data give some evidence that enamel has this property. Contrary to the findings of this report, however, Perry and Armstrong ('41) found no increase in fluorine in erupted rat molar dentine. Perhaps the age of their rats (mature male rats averaging 250 gm.) and the duration of their experiment (60 days) may account for the different results obtained.

Post-eruptive deposits of fluorine may not increase the tooth's caries resistance. Thus, although fluorine increased in the molars of injected rats, caries remained the same as in control rats. A similar result applies to rats receiving 5 ppm. of fluorine in water. Also the fluorine in the molars of three groups of rats given 10 ppm. fluorine water is not proportional to the observed caries prevention. At the present time, therefore, it seems unlikely that these secondary post-eruptive

deposits of fluorine exerted a significant effect on the development of rat caries. Except for the injection group, perhaps any effect of tooth fluoride may have been masked by food or water-borne fluoride.

#### SUMMARY

A minimum of 10 ppm. of fluorine in drinking water gave partial protection against occlusal rat caries. Approximately 80% protection resulted from both 50 ppm. and 100 ppm. fluorine in water. It is suggested that fluorine acts to inhibit rat caries by anti-enzymatic local action within the oral cavity.

The pooled molar teeth of groups of carious rats were not different from those of non-carious rats in content of ash, calcium, and phosphorus, and in fluorine which was deposited post-eruptively.

The post-eruptive addition of fluorine to rats' molar teeth occurred in relatively large quantities and is regarded as occurring independent of oral enamel surface adsorption of fluoride. A significant effect of fluoride acquired after tooth eruption on induced rat caries was not evident.

#### LITERATURE CITED

- ARMSTRONG, W. D., AND P. J. BREKHUS 1938 Possible relationship between the fluorine content of enamel and resistance to dental caries. *J. Dent. Res.*, vol. 17, p. 393.
- ARNOLD, F. A., JR., AND F. J. MCCLURE 1941 Observations on rat caries. II. Effect of injected sodium fluoride on induced rat caries. *J. Dent. Res.*, in press.
- CHEYNE, V. D. 1940 Inhibition of experimental dental caries by fluorine in the absence of saliva. *Proc. Soc. Exp. Biol. and Med.*, vol. 43, p. 58.
- COX, G. J., M. L. DODDS, S. F. DIXON AND M. C. MATUSCHAK 1939 Experimental dental caries. III. A system of recording corn-meal caries in rats. *J. Dent. Res.*, vol. 18, p. 469.
- COX, G. J., M. C. MATUSCHAK, S. F. DIXON, M. L. DODDS AND W. E. WALKER 1939 Experimental dental caries. IV. Fluorine and its relation to dental caries. *J. Dent. Res.*, vol. 18, p. 481.
- DEAN, H. T. 1938 Endemic fluorosis and its relation to dental caries. *Pub. Health Rep.*, vol. 53, p. 1443.
- DEAN, H. T., P. JAY, F. A. ARNOLD, JR., F. J. MCCLURE AND E. ELVOVE 1939 Domestic water and dental caries including some epidemiological aspects of *L. acidophilus*. *Pub. Health Rep.*, vol. 54, p. 862.

- DEAN, H. T., P. JAY, F. A. ARNOLD, JR. AND E. ELVOVE 1941 b Domestic water and dental caries. II. A study of 2832 white children age 12-14 years, of 8 suburban Chicago communities including Lactobacillus acidophilus studies of 1761 children. *Pub. Health Rep.*, vol. 56, p. 761.
- 1941 a I. A dental caries study including *L. acidophilus* estimations of a population severely affected by mottled enamel and which for the past twelve years have used a fluoride free water. *Pub. Health Rep.*, vol. 56, p. 365.
- DONALDSON, H. H. 1934 On the increase in the diameters of the molar teeth of the rat after eruption. *J. Dent. Res.*, vol. 14, p. 323.
- FINN, S. B., AND H. C. HODGE 1938 The inhibition of experimental dental caries by fluoride. *J. Dent. Res.*, vol. 18, p. 252.
- HOFFMAN, M. M., AND I. SCHOUR 1940 Quantitative studies in the development of the rat molar. I. The growth pattern of the primary and secondary dentine (from birth to 500 days of age). *Anat. Rec.*, vol. 78, p. 233.
- LILLY, C. A., AND L. WILEY 1934 Relation between the physical character of food and dental caries in albino rats. *J. Nutrition*, vol. 7, p. 463.
- LUNDSGAARD, E. 1930 Untersuchungen muskelkontraktionen ohne Milch Saure Bildung. *Biochem. Zeit.*, vol. 217, p. 102.
- MANLEY, R. S., AND H. C. HODGE 1939 Density and refractive index studies of dental hard tissues. II. Methods for separation and determination of purity. *J. Dent. Res.*, vol. 18, p. 133.
- MILLER, B. F. 1938 Inhibition of experimental dental caries in the rat by fluoride and iodoacetic acid. *Proc. Soc. Exper. Biol. and Med.*, vol. 39, p. 389.
- McCLURE, F. J. 1939 a Microdetermination of fluorine by thorium nitrate titration. *Ind. and Eng. Chem. Analytical Ed.*, vol. 18, p. 171.
- 1939 b Fluorides in food and drinking water. A comparison of effects of water-ingested versus food-ingested sodium fluoride. U. S. Public Health Service, National Institute of Health Bulletin No. 172, pp. 53.
- 1941 Domestic water and dental caries. III. Fluorine in human saliva. *Am. J. Diseases of Children*. Accepted for publication.
- McCLURE, F. J., AND F. A. ARNOLD, JR. 1941 Observations on induced dental caries in rats. I. Reduction by fluorides and iodoacetic acid. *J. Dent. Res.*, vol. 20, p. 97.
- PERRY, M. W., AND W. D. ARMSTRONG 1941 On the manner of acquisition of fluorine by mature teeth. *J. Nutrition*, vol. 21, p. 35.
- ROBISON, R., AND A. H. ROSENHEIM 1934 Calcification of hypertropic cartilage in vitro. *Biochem. J.*, vol. 28, p. 684.
- "STUDENT" 1925 New tables for testing the significance of observations. *Metron*, vol. 5, p. 105.
- VOLKER, J. F. 1939 Effect of fluoride on solubility of enamel and dentine. *Proc. Soc. Exper. Biol. and Med.*, vol. 42, p. 725.
- WILLS, J. H. 1940 The secretion of intravenously injected fluorine in the submaxillary saliva of cats. *J. Dent. Res.*, vol. 19, p. 585.
- WOOD, H. E. 1935 Do rat molars grow diametrically after eruption? *J. Dent. Res.*, vol. 15, p. 9.

## STUDIES ON THE RAT GROWTH ASSAY METHOD FOR RIBOFLAVIN

HAROLD R. STREET

*Research Laboratories, Winthrop Chemical Company, Inc., Rensselaer, New York*

ONE FIGURE

(Received for publication April 7, 1941)

The recent status of the rat growth procedure for the assay of riboflavin was adequately summarized by Wagner, Axelrod, Lipton and Elvehjem ('40). This report indicates that the older diets, such as the widely used Bourquin-Sherman ('31) ration, appear to be inadequate in certain factors necessary for optimal nutrition of the rat, exclusive of riboflavin. More recently diets have been described which support nearly normal growth when supplemented only with riboflavin. Some of the materials that have been proposed to supply the vitamin B complex in these diets are charcoal-treated liver extract (El Sadr, Macrae and Work, '40), fuller's earth-treated rice bran extract (Clarke, Lechycka and Cook, '40), fuller's earth-treated yeast extract (Hunt and Bethke, '40), and photolyzed liver concentrate plus thiamine and vitamin B<sub>6</sub> (Wagner, Axelrod, Lipton and Elvehjem, '40).

In the author's experience, an untreated rice bran extract has been found to be a satisfactory source of the B vitamins, other than riboflavin, needed by the rat and thus suitable for the preparation of a standard riboflavin-low basal diet. Extensive experiments involving more than 1500 rats have shown that the ration proposed permits a high degree of assay accuracy. It is believed that the diet thus developed offers certain distinct advantages as to simplicity and ease of preparation over other riboflavin-deficient diets that have been described.

## EXPERIMENTAL

Young male rats from our laboratory colony, weighing 40 to 55 gm., are placed in individual wire cages in an air-conditioned room, the temperature of which is kept at  $77^{\circ} \pm 2^{\circ}$ F. The diet used is a modification of that described by Street and Reeves ('40) and consists of a vitamin B complex-free basal mixture fed ad libitum, supplemented at standard time intervals with rice bran extract. The basal mixture has the following percentage composition: vitamin-free casein<sup>1</sup> 18; cornstarch 63; hydrogenated cottonseed oil<sup>2</sup> 12; cod liver oil 3; and salt mixture (no. 2 of U. S. P. XI) 4. In addition to this ration, each rat is given an aqueous extract of rice bran<sup>3</sup> three times weekly, fed in small supplement dishes, at a level supplying 15 µg. of thiamine per rat daily. This level of supplement has proved adequate for test purposes, since with the addition of optimal amounts of riboflavin it permits a very satisfactory, although slightly subnormal, growth rate on the basis of data from our colony (fig. 1). However, it might be advisable for very rapidly growing strains of rats to increase somewhat the amount of the extract given. In place of the above supplement one may use with equal success the 25% alcoholic extract of rice bran described by Street and Cowgill ('39) at a level equivalent to 1 gm. rice bran per rat daily, as indicated by Street and Reeves ('40).

The animals are weighed twice weekly starting 1 to 2 weeks after the beginning of the depletion period. They are considered ready for testing when the weight gains approximate 4 gm. or less in the last 10 days; but animals gaining up to 6 gm. in 10 days are also considered satisfactory for assay purposes if they have not gained more than 2 gm. during the last week. The depletion period on the above diet at

<sup>1</sup> Labco brand.

<sup>2</sup> Crisco.

<sup>3</sup> Vitab Rice Bran Concentrate, obtainable from the National Oil Products Company, Inc., Harrison, New Jersey. This material "is an aqueous extract of rice bran concentrated to a specific gravity of 1.38 and clarified by filtration." The vitamin content of this preparation is constant over long periods of time when it is stored under refrigerator conditions.

certain seasons of the year has been found to be 2 to 4 weeks; at other seasons, 3 to 5 weeks, as indicated in table 2. There are usually a few animals, never more than 10% of the total, which continue to gain after the weights of the others have reached a plateau. These exceptional animals are discarded. Test groups of ten or more animals are used for both assay and control purposes. Three control groups are used for each assay and include a group of negative controls to which no riboflavin is given, a second group with a supplement of 5  $\mu\text{g}$ . of crystalline riboflavin per day, and a third group which receives 10  $\mu\text{g}$ . of riboflavin per day. The 7-day dosage supplement is given on a 6-day basis, i.e., each day-dose represents one-sixth the amount consumed weekly. However, experience has indicated that the test fractions may be given three times weekly in twice the calculated day-dose. This sequence, as recommended by Carlsson and Sherman ('38), is entirely satisfactory and economical as to time. The standard solution of riboflavin or the preparations to be assayed are measured into individual supplement dishes containing the basal rice bran extract supplement.

The test and control animals are weighed weekly during the period of assay and the average gain in weight for this 4-week period is calculated for each group. The growth of the negative control animals on the basal ration alone averages 6 to 9 gm. in 4 weeks, but the maximal range is 0 to 12 gm. The growth induced by various riboflavin dosage levels under the above test conditions is shown in table 1.

The results have been expressed in terms of the effective gain, that is, the gain of any group minus the gain of the negative controls. It may be noted that, at a given season of the year, the weight gain per unit dosage of riboflavin is the same within the limits of 2.5 and 10  $\mu\text{g}$ . of the vitamin per day.

The growth response to varying amounts of riboflavin throughout a 6-week period following depletion is shown graphically in figure 1. The gain of 70 to 80 gm. in 4 weeks induced by a supplement of 50  $\mu\text{g}$ . of riboflavin daily ap-

proaches the normal growth rate, since the rats of this colony usually gain about 100 gm. in 4 weeks on a stock ration.

In calculating the results of an assay the effective gain per microgram of riboflavin is obtained from the performance of the control groups at the 5 and 10 µg. levels, and from this figure the riboflavin content of the day-dose of the unknowns is calculated. These procedures permit an evaluation of unknown substances administered at a single dose level, providing the actual content of the day-dose lies between 2.5 and 10 µg. (The response at higher levels, although materially greater, has not been investigated on a quantitative basis.)

TABLE 1  
*The rate of growth induced by supplementing the basal diet with various amounts of riboflavin.*

ASSAY NO.	DATE BEGUN	NO. OF RATS USED	DAILY DOSE OF RIBOFLAVIN	EFFECTIVE GAIN IN 4 WEEKS <sup>1</sup>	GAIN PER MICROGRAM OF RIBOFLAVIN
Assays conducted in 1939					
4	May	9	6.25	27.5	4.4
		10	7.50	29.3	3.9
		10	8.75	36.4	4.2
5	June	9	7.5	34.2	4.6
6	June	10	5.0	20.1	4.0
		9	7.5	28.3	3.8
		10	10.0	42.1	4.2
Assays conducted in 1940					
14	January	10	2.5	8.1	3.2
		11	5.0	18.2	3.6
15	March	10	5.0	17.6	3.5
		10	10.0	33.8	3.4
16	April	11	5.0	18.6	3.7
		11	10.0	37.7	3.8
17	May	10	5.0	20.9	4.2
		10	10.0	41.9	4.2
18	June	9	5.0	22.6	4.5
		9	10.0	44.8	4.5
19	July	9	2.5	11.3	4.5

<sup>1</sup> The effective gain indicates the total gain in weight of any group minus the gain of the negative controls.

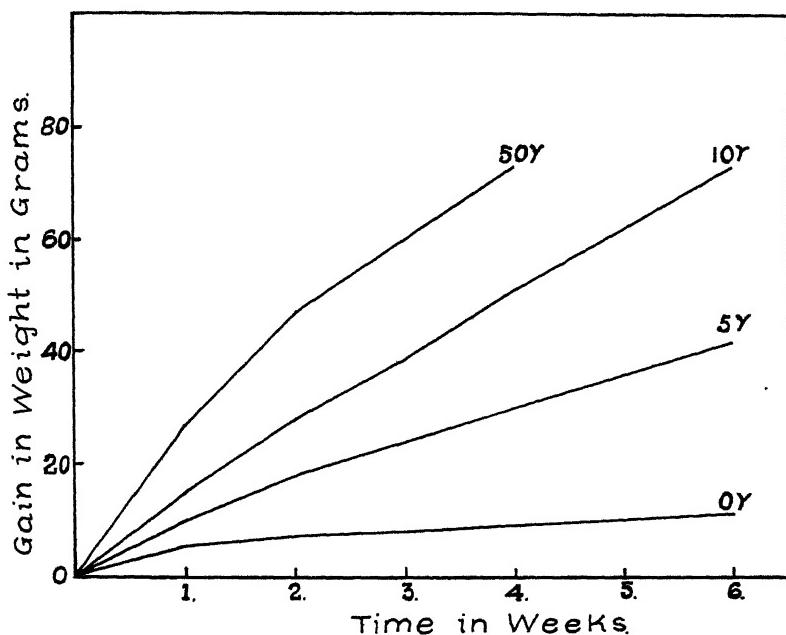


Fig. 1 Graph showing the growth induced by supplementing the basal diet with various levels of riboflavin. The figure above each curve indicates the daily dose of riboflavin in micrograms.

TABLE 2  
*Seasonal variation in depletion period.*

ASSAY NO. <sup>1</sup>	DATE BEGUN	MEAN LENGTH OF DEPLETION PERIOD	ASSAY NO. <sup>1</sup>	DATE BEGUN	MEAN LENGTH OF DEPLETION PERIOD
	1939	<i>days</i>		1940	<i>days</i>
4	May	34.5	13	January	18.5
5	June	34.6	14	January	16.6
6	June	31.0	15	March	17.5
7	July	33.5	16	April	17.9
8	August	31.4	17	May	20.8
9	September	26.4	18	June	23.5
10	October	21.9	19	July	24.8
11	November	19.2	20	September	21.9
12	December	19.9	21	November	18.0
			22	December	17.0

<sup>1</sup> Each assay represents sixty to eighty male rats.

*Seasonal variations in results.* Despite the fact that the stock diet used in our rat colony is of uniform composition, there is a definite seasonal variation both in the time required for depleting the animals of riboflavin and in the effective gain per unit of riboflavin. These variations are shown in tables 1 and 2. It will be observed that the necessary depletion period is longer in spring and summer. During this period the animals also make larger gains in weight.

*Validity of the procedure.* It is obvious that a partial deficiency of the basal or test diets in any essential factor other than riboflavin would lead to a greater gain per unit of riboflavin, as represented by natural foods, than for pure riboflavin, since the food supplement could be expected to supply the other factors present in the basal diet in sub-optimal amounts. The results of assays of several lots of a vitamin B complex elixir, added as supplements to the suggested test procedures, are shown in table 3.

TABLE 3  
*Riboflavin assays of vitamin B complex elixir.*

SAMPLE NO.	ACTUAL RIBOFLAVIN CONTENT	RIBOFLAVIN CONTENT FOUND BY ASSAY	
		Absolute content	Per cent of true value
—	μg./cc.	μg./cc.	%
1	25	24.5	98
2	25	26.5	106
3	25	27.8	111
4	25	25.3	101
5	25	25.8	103
6	25	27.2	109
7	25	24.8	99
8	25	27.2	109
9	25	24.2	97
10	25	29.8	119
11	25	25.0	100

In this preparation the riboflavin content has been fortified by means of the synthetic material, so that only 20% of the total present is represented by the natural riboflavin contained in rice bran extract. Thus, the actual riboflavin content is known with considerable exactness. It will be observed that

the method applied to this material shows approximately the expected content, indicating that the various water-soluble vitamins other than riboflavin present in rice bran extract do not appreciably affect the growth rate.

As a further check on the validity of the method assays were made with a preparation of known riboflavin content. Filtrate factor free of riboflavin and of vitamins B<sub>1</sub> and B<sub>6</sub> was prepared by treating rice bran extract five times with fuller's earth at pH 4.0. This filtrate factor was then fortified with synthetic vitamins in such amounts that the final preparation contained 10 µg. of riboflavin, 30 µg. of thiamine, and 25 µg. of vitamin B<sub>6</sub> per cubic centimeter. Another preparation containing approximately 10 µg. of riboflavin per cubic centimeter was prepared by adding a solution of riboflavin to rice bran extract, so that the final solution contained per cubic centimeter 2 µg. of riboflavin from the extract and 8 µg. of synthetic riboflavin. The results of assays of these two preparations are shown in table 4.

TABLE 4

*Assay of complex preparations containing 10 µg. of riboflavin per cubic centimeter.*

GROUP NO.	SUPPLEMENT	ACTUAL RIBOFLAVIN CONTENT OF DAILY DOSE µg.	AVERAGE WEIGHT GAIN IN 4 WEEKS			APPARENT RIBOFLAVIN CONTENT OF DAILY DOSE µg.
			Total gain gm.	Gain over negative controls gm.		
1	None	0	7.7			
2	Riboflavin	2.5	19.0	11.3		
3	Rice bran extract plus riboflavin	5.0	33.4	25.7	5.7	
4	Filtrate factor plus riboflavin, thiamine, and vitamin B <sub>6</sub>	5.0	31.7	24.0	5.3	

The effective gain (growth of assay group minus growth of negative controls) of 11.3 gm. in 4 weeks for the control group receiving a 2.5 µg. dose of riboflavin is equivalent to 4.52 gm. per microgram of riboflavin. From this figure the apparent riboflavin content of the daily dose has been calculated for groups 3 and 4. It will be seen that the observed value of the

fortified filtrate factor, the riboflavin content of which was known with exactness, was only 6% greater than the true value.

These data appear to indicate that the use of the assay procedure described permits satisfactory determinations of the riboflavin contained in natural products.

*Experiments with wheat extract as a source of the vitamin B complex.* Frequently large discrepancies exist between riboflavin assays of products by different laboratories. Presumably these variations are to a considerable extent due to differences in the diets used. To determine more definitely the difference that might be expected because of variation in diet, the author's procedure has been compared directly with the original Bourquin-Sherman method, which utilizes an 80% alcoholic extract of wheat as the source of the vitamin B complex of the diet. The result of the assay of substances of known riboflavin content by this procedure is shown in table 5.

TABLE 5

*Experiments with a ration containing wheat extract as a source of the vitamin B complex.*

GROUP NO.	SUPPLEMENT	ACTUAL RIBOFLAVIN CONTENT OF DAILY DOSE μg.	AVERAGE WEIGHT GAIN IN 3 WEEKS	
			Total gain gm.	Gain over negative controls gm.
1	None	0	-3.7	
2	Synthetic riboflavin	2.5	2.8	6.5
3	Synthetic riboflavin	5.0	5.7	9.4
4	Synthetic riboflavin	10.0	9.0	12.7
5	Vitamin B complex elixir, sample 2	2.5	13.8	19.5
6	Filtrate factor plus riboflavin, thiamine, and vitamin B <sub>6</sub>	2.5	17.4	21.1
7	English ale yeast	2.5	12.6	16.3

The elixir and the fortified filtrate factor correspond to those previously tested (tables 3 and 4). The value expressed for the English ale yeast was obtained by the author's assay procedure. The data presented have been calculated on the basis of the 3-week gains since the flattening of the growth curve mentioned by Sherman and Smith ('31) appeared after the third week in this experiment.

It may be observed that the gain per unit of riboflavin was very much greater for the preparations tested than for the pure vitamin. Thus, for groups 5, 6 and 7 receiving 2.5 µg. of riboflavin per day in preparations containing natural products, the effective gain was two to three times that for group 2, which received 2.5 µg. of synthetic riboflavin per day. These results indicate clearly that it is unsafe to conclude that a particular lot of a diet may be safely used for riboflavin assay merely because it permits increasing growth with increasing amounts of riboflavin at low levels of supplementation.

#### DISCUSSION

The results of the experiment on the use of wheat extract are in general agreement with those of Schumaker and Heuser ('40) who found that instead of using the customary conversion factor of 2.5 µg. of riboflavin per Bourquin and Sherman unit, the true value was obtained with a conversion factor of 2.0 µg. per unit for residual yeasts and 1.3 µg. for primary grown yeasts. They took as the true riboflavin content their findings with a photometric procedure and with a chick assay procedure. Likewise Clarke and coworkers ('40) have found wheat extract to be unsatisfactory as a source of the B complex in riboflavin assays because of the presence of only sub-optimal amounts of certain factors other than riboflavin.

With the diet and procedure described in this paper there is a linear relation between the growth response, expressed as the gain over the negative controls, and the dose of riboflavin administered. In contrast, Clarke and coworkers ('40) under the conditions of their procedure have found a linear relation to exist between the total gain and the logarithm of the dose administered. The author's data cannot be made to conform to linearity when similarly expressed, but the relations between dose and response are in close agreement with those presented by Sherman and Smith ('31) for the Bourquin-Sherman diet when the riboflavin is furnished by natural foods. Likewise, Hunt and Bethke ('40) using a diet containing fuller's earth-treated yeast as a source of the water-soluble vitamins, found a linear relation between the dose

of riboflavin and the growth expressed as the increase in weight over the negative controls. Similarly, Wagner, Axelrod, Lipton and Elvehjem ('40) have found that with their riboflavin deficient ration, the relation between growth and riboflavin administered is approximately linear over the range of 3 to 9  $\mu\text{g}$ . of riboflavin per day.

#### SUMMARY

Since diets complete in all the factors required by the rat other than riboflavin have been described, there would seem to be little reason for the continued use of rations containing wheat extract, in that wheat does not appear to be an adequate source of the vitamin B complex for such purposes.

The diet and assay procedure described, in which an untreated rice bran extract is utilized for supplement purposes, represent certain improvements over available test methods and permit a rapid, accurate, and economical assay of riboflavin from either synthetic, natural or mixed (pharmaceutical) sources.

#### LITERATURE CITED

- BOURQUIN, A., AND H. C. SHERMAN 1931 Quantitative determination of vitamin G(B<sub>2</sub>). *J. Am. Chem. Soc.*, vol. 53, pp. 3501-3505.
- CARLSSON, E. V., AND H. C. SHERMAN 1938 Riboflavin and a further growth essential in the tissues. *J. Nutrition*, vol. 15, pp. 57-65.
- CLARKE, M. F., M. LECHYCKA AND C. A. COOK 1940 The biological assay of riboflavin. *J. Nutrition*, vol. 20, pp. 133-144.
- EL SADR, M. M., T. F. MACRAE AND C. E. WORK 1940 The estimation of riboflavin. Part I. A new biological method. *Biochem. J.*, vol. 34, p. 601.
- HUNT, C. H., AND R. M. BETHKE 1940 The riboflavin content of certain hays and grasses. *J. Nutrition*, vol. 20, pp. 175-180.
- SCHUMAKER, A. E., AND G. F. HEUSER 1940 Riboflavin content of yeasts determined photometrically and biologically. *Ind. and Eng. Chem., Anal. Ed.*, vol. 12, pp. 203-204.
- SHERMAN, H. C., AND S. L. SMITH 1931 The vitamins. *Chemical Catalog Company*, New York.
- STREET, H. R., AND G. R. COWGILL 1939 Acute riboflavin deficiency in the dog. *Am. J. Physiol.*, vol. 125, pp. 323-334.
- STREET, H. R., AND R. E. REEVES 1940 Occurrence of riboflavin in tubercle bacillus. *Proc. Soc. Exper. Biol. and Med.*, vol. 44, pp. 641-644.
- WAGNER, J. R., A. E. AXELROD, M. A. LIPTON AND C. A. ELVEHJEM 1940 A rat assay method for the determination of riboflavin. *J. Biol. Chem.*, vol. 136, pp. 357-364.

# THE NICOTINIC ACID AND COENZYME CONTENT OF THE TISSUES OF NORMAL AND BLACKTONGUE DOGS

W. J. DANN AND PHILIP HANDLER

*Department of Physiology and Pharmacology, Duke University School of Medicine,  
Durham, North Carolina*

(Received for publication June 18, 1941)

It has been concluded by Kohn, Klein and Dann ('39) and by Axelrod, Madden and Elvehjem ('39) that the coenzyme content of the liver and voluntary muscle of the dog is decreased in blacktongue (nicotinic acid deficiency). Neither group of investigators produced evidence which proved this point conclusively; the figures of the former authors showed too great a variation within each of the groups of dogs compared and the latter gave figures only for one normal and two blacktongue dogs. It was, therefore, decided to reinvestigate the problem more thoroughly, and in addition to the coenzyme comparison to perform parallel tissue analyses for nicotinic acid.

## EXPERIMENTAL

Blacktongue was induced in a series of dogs by maintaining them on the nicotinic acid deficient regimen described by Kohn, Klein and Dann ('39), modified by altering the water-soluble vitamin supplement from 1 mg. thiamine chloride weekly to 1 mg. thiamine chloride and 1 mg. riboflavin twice weekly. The normal dogs used for comparison were kept on the stock diet described by the same authors.

The total pyridine nucleotides (coenzymes I and II) were estimated as V-factor by the method of Kohn ('38). The validity of this procedure has been confirmed by Handler and

Dann ('41) using the tissues of the rat. The tissue extracts were compared with solutions of a standard preparation of diphosphopyridine nucleotide obtained from yeast by the method of Warburg and Christian ('36). This standard was determined to be 75% pure on the basis of its nicotinic acid and phosphorus content. Nicotinic acid analyses were performed by the method of Dann and Handler ('41a). All the dogs were decapitated for maximum exsanguination and tissue samples were taken for analysis within 15 minutes after death. The blacktongue dogs were sacrificed when they were so severely ill that death seemed certain within 24 hours.

Table 1 summarizes the nicotinic acid and coenzyme content of three tissues of ten normal dogs. The coenzyme content is expressed as micrograms of diphosphopyridine nucleotide equivalent in activity to 1 gm. of fresh tissue. This method of expression is valid because the di- and triphosphopyridine nucleotides have the same growth stimulating action on *H. parainfluenzae* in equimolar concentrations. By "bound"

TABLE 1  
*Nicotinic acid and coenzyme I and II content of tissues of dogs.*

AN- MALS	FACTORS DETER- MINED	LIVER			KIDNEY			MUSCLE		
		Mean & S.E.	Range	Per cent of normal	Mean & S.E.	Range	Per cent of normal	Mean & S.E.	Range	Per cent of normal
Ten normal dogs	Coenzyme I & II	438±46	290-720	504±18	388-570	310±12	250-356			
	Total NA	153±12	90-233	95±3.4	75-117	71±1.9	59-80			
	Bound NA	81±8.5	53-133	98±3.8	72-106	57±2.2	46-66			
	Unbound NA	72		0		14				
Ten black- tongue dogs	Coenzyme I & II	184±19	114-309	42	500±15	416-564	100	222±28	86-360	72
	Total NA	101±4.3	84-117	66	96±2.7	75-104	100	59±5.3	32-81	83
	Bound NA	34±3.5	21-57		92±2.8	77-104		41±5.2	16-60	
	Unbound NA	67		93	0			? <sup>1</sup>		? <sup>1</sup>
Four normal dogs with excess nicotinic acid	Coenzyme I & II	457	371-526		461	405-432		342	319-360	
	Total NA	151	142-159		91	86-94		77	72-86	
	Bound NA	85	70-97		85	75-98		64	59-67	
	Unbound NA	66			? <sup>1</sup>			13		

<sup>1</sup> The query sign indicates that a figure could be entered by calculation from other data given, but it would have no statistical significance.

nicotinic acid is meant only that nicotinic acid bound as coenzyme; this figure is obtained on dividing the quantity of coenzyme by 5.4. By "unbound" nicotinic acid is meant all nicotinic acid other than that which is bound in coenzymes I and II.

Almost half of the nicotinic acid present in the livers of the normal dogs was found to be unbound, as was 20% of the nicotinic acid of the muscle. No unbound nicotinic acid appeared to be present in the kidney cortex. Unlike the dog, neither rat kidney cortex nor rat muscle contains any unbound nicotinic acid (Handler and Dann, '41). The statistical significance of the figures is indicated by the standard errors of the means and the ranges reported.

Table 1 also gives corresponding figures for dogs with severe blacktongue. As with the normal animals, the livers contained unbound nicotinic acid and the kidney cortex none; but the situation in muscle is uncertain. The figures given suggest that 10-20% of the nicotinic acid was unbound, as in normal dogs, but do not establish this point conclusively because the difference of the means of total and bound nicotinic acid was not significant owing to large variations within each group. About 30% of the liver nicotinic acid was unbound.

The columns in table 1 which are headed "per cent of normal" give a summary comparison of the tissues of normal and blacktongue dogs. In blacktongue the greatest change from the normal occurred in the liver; the total nicotinic acid content decreased to 66% of the normal and almost all of this decrease occurred in the bound fraction of the nicotinic acid. The coenzyme level (and, therefore, the bound nicotinic acid also) decreased to 42% of the normal, but the unbound nicotinic acid fell only to 93% of the normal.

In the skeletal muscle examined, similar but less pronounced changes occurred; they were not so clear-cut because the variation within the blacktongue group was greater, but there was a significant decrease both in the total nicotinic acid and in the coenzyme content. Again it appeared that only the bound fraction of the nicotinic acid decreased. Although the per-

centage decrease in the muscle was less than that in liver, considerably more nicotinic acid was lost from the musculature than from the liver owing to the difference between their weights. No change was observed in the kidney cortex.

Additional observations were made on dogs fed an excess of nicotinic acid, to determine the effect on the tissue content. Four dogs were maintained on the stock diet and given 20 mg. of nicotinic acid subcutaneously each day for 2 months. They were then decapitated and the tissues sampled for analysis. The results of these analyses are collected in table 1 and show no significant difference from the corresponding figures for normal dogs either in coenzyme or total nicotinic acid content. From this it appears that the dog is unable to store notable quantities of nicotinic acid or the pyridine nucleotides, and in this it resembles the rat (Axelrod, Madden and Elvehjem, '39; Dann and Handler, '41 b).

#### DISCUSSION

The findings reported above substantiate the conclusion of Kohn, Klein and Dann ('39) that there is a significant decrease from the normal in the coenzyme content of the liver and muscle of blacktongue dogs, although the decrease is not quite as great as they reported. Instead of a decrease in liver to 30% and in muscle to 65% of the normal, we now find decreases to 42% and 72% respectively. The figures given by Axelrod, Madden and Elvehjem ('39) also suggested such decreases, but the absolute values reported were almost twice as great as ours. Since there is not enough nicotinic acid present in the tissues to account for their high levels of coenzyme, it appears likely that their figures were too high. Such an error could arise either in the calibration of their yeast preparation or from incorrect evaluation of the purity of the standard diphosphopyridine nucleotide preparation with whose activity the tissues were compared. It has been pointed out by Handler and Dann ('41) that the latter is the more probable explanation. Recently, Axelrod, Spies and Elvehjem ('41) found a decrease from the normal in the coenzyme content of human

muscle in pellagra, corresponding to that found in canine blacktongue.

The suggestion has been made (Stannus, '40) that pellagra is due to impairment of the oxidizing systems of the cells, in turn due to decrease of the coenzyme level. The evidence of Kohn, Klein and Dann ('39) showed that this is unlikely, so far as known respiratory systems are concerned. Such a hypothesis takes no account of the tenacity with which the unbound nicotinic acid is retained in certain organs even when the coenzyme level falls; it is to be expected that nicotinic acid so retained has an equally vital function, although it is not possible to assign this function at present.

As it seems unlikely that the loss of coenzymes I and II from the body is the direct cause of the onset of pellagra or blacktongue and of death from these diseases, the question of the immediate cause of death remains unsolved. Observation of the experimental animals suggests that dehydration or the toxic reactions of the inflammation following the invasion of the alimentary mucous membranes may be the cause. Further experiments are now in progress to test the effect in acute blacktongue of (a) maintenance of body fluids by parenteral administration and (b) attack on the organisms invading the mucous membranes with chemo-therapeutic agents.

Our thanks are due to the John and Mary R. Markle Foundation for a grant in aid of this study; and to Merck and Company for the thiamine and riboflavin used.

#### LITERATURE CITED

- AXELROD, A. E., R. J. MADDEN AND C. A. ELVEHJEM 1939 The effect of a nicotinic acid deficiency upon the coenzyme I content of animal tissues. *J. Biol. Chem.*, vol. 131, p. 85.
- AXELROD, A. E., T. D. SPIES AND C. A. ELVEHJEM 1941 The effect of a nicotinic acid deficiency upon the coenzyme I content of human erythrocyte and muscle. *J. Biol. Chem.*, vol. 138, p. 667.
- DANN, W. J., AND P. HANDLER 1941 a The estimation of nicotinic acid in animal tissues. *J. Biol. Chem.*, vol. 140, p. 201.  
——— 1941 b Unpublished data.

- HANDLER, P., AND W. J. DANN 1941 The nicotinic acid and coenzyme content of animal tissues. *J. Biol. Chem.*, vol. 140, p. 739.
- KOHN, H. I., J. R. KLEIN AND W. J. DANN 1939 The V-factor content and oxygen consumption of the tissues of the normal and blacktongue dog. *Biochem. J.*, vol. 33, p. 1432.
- KOHN, H. I. 1938 The concentration of coenzyme-like substances in blood following the administration of nicotinic acid to normal individuals and pellagrins. *Biochem. J.*, vol. 32, p. 2075.
- STANNUS, H. S. 1940 Pellagra. *Lancet*, vol. 1, p. 352.
- WARBURG, O., AND W. CHRISTIAN 1936 Pyridin, der wasserstoffübertragende Bestandteil von Gärungsfermenten. *Biochem. Z.*, vol. 287, p. 291.

# THE MINIMUM REQUIREMENT OF RABBITS FOR dl- $\alpha$ -TOCOPHEROL

SAMUEL H. EPPSTEIN AND SERGIUS MORGULIS

*Department of Biochemistry, University of Nebraska,  
College of Medicine, Omaha*

TWO FIGURES

(Received for publication June 4, 1941)

Little is known concerning the nutritional requirements of various species for vitamin E. Since pure  $\alpha$ -tocopherol, the most potent of the compounds having E activity, has become available the measurements of the need for this vitamin are now feasible. When sufficient data have been accumulated, perhaps certain generalizations will then be possible.

Evans and Emerson ('39) state that 3 mg. of  $\alpha$ -tocopherol will allow a young mature female rat to bear a normal litter and that 0.75 mg. daily will protect the male rat against testicular degeneration (Evans, Emerson and Emerson, '39). Mackenzie and McCollum ('40) have reported that the rabbit needs no more than 1 mg. daily per kilogram of body weight. The present communication reports the results of experiments on the minimum quantities of dl- $\alpha$ -tocopheryl acetate needed to cure and maintain in good health rabbits suffering from nutritional muscular dystrophy caused by E-avitaminosis. Other factors, such as the method of administration, effect of sex and age will also be discussed. A preliminary report of this work has been published elsewhere (Eppstein and Morgulis, '40).

## EXPERIMENTAL

Albino rabbits weighing 400 to 600 gm. were employed in these experiments. Weighings were made daily, and food and water given ad libitum. The progress of the dystrophy was

followed by growth curves and daily clinical observation. By these means an experienced observer can appraise the various stages of dystrophy as well as when the degree of creatinuria is included. This method has the advantage of requiring little time and, by dispensing with metabolism cages, it also permits the use of large numbers of animals for the tests. Biopsies on the gastrocnemius muscle were made in about half the cases as a further check on the degree of dystrophy. The microscopic examination and clinical findings were in good agreement. It should be emphasized that before cure was attempted the deficiency of E was maintained until the dystrophic condition became very definite as was determined by posture, sensitivity of muscles, stiffness, muscle tone, and by the ability of the animal to right itself from a supine position. This stage always occurred almost simultaneously with the downward break in the growth curve, the two occurring within 2 or 3 days of each other. Both criteria of the onset of dystrophy had to be fulfilled, however, before curative treatment was started. The expression "cure of muscle dystrophy," we believe, should only be used when dealing with animals showing active signs of the disease, and not merely suggestive symptoms. The interval of time during which a given supplement offered protection was determined by noting when the dystrophic symptoms reappeared.

Diet 113X had the following percentage composition: extracted wheat germ, 60; extracted casein, 15; extracted yeast, 5; sucrose, 7; salt mixture (McCollum's 185, modified by the addition of 45 gm. CaCO<sub>3</sub> to 80 gm. of the mixture), 3; lard, 8; and cod liver oil, 2%. The wheat germ was exhaustively extracted with hot, dry acetone. The casein and yeast were extracted seven times at room temperature by covering with petroleum ether (2 liters per kilogram of solids) for 12-hour periods. The lipid residues in these extracted materials were as follows: wheat germ about 2 mg. %; casein 50 mg. %; and yeast about 20 mg. %, or a total of 10-15 mg. of lipid from these sources per animal per day. For comparative purposes some animals were fed the Goettsch and Pappenheimer diet 13

(Goettsch and Pappenheimer, '31). Of the completely mixed diets (13 and 113X) enough was prepared at one time to last only 4-5 days. The supplies were kept in tightly-closed cans.

Appropriate amounts of dl- $\alpha$ -tocopheryl acetate<sup>1</sup> were dissolved in olive oil or in sesame oil and stored in brown bottles under CO<sub>2</sub> at 4°C. The dose of the supplement (always contained in 1 ml.) was given per os from a Mohr pipette calibrated with the oil solution. The rabbits, made dystrophic on either of the two diets, were fed graded doses of the ester ranging from 0.25 mg. to 6 mg. per day (calculated as the free tocopherol). The tocopherol was usually given daily but in some cases the appropriate multiple dose was fed at 2, 4, or 5 day intervals. Sometimes a 1 to 5 mg. dose was given and when the roughly calculated period of protection was almost up a second dose was fed, etc. The supplementation was continued for periods up to 45 days, and the length of time after its discontinuance until the first symptoms of dystrophy reappeared was noted. In a few instances the protection resulting from single massive doses of dl- $\alpha$ -tocopheryl acetate was studied. The minimum daily requirement for each animal was then calculated by dividing the total amount of the acetate fed (expressed as tocopherol) by the summation of the daily weights in kilograms from the time supplementation was begun until the animal showed signs of recurrent dystrophy. This method takes into account the variations in body weight and the results are expressed in milligrams per kilogram of body weight per day. Since it has been demonstrated that the racemic product is as effective as the naturally occurring tocopherol (Karrer, Fritzsche, Ringier and Salomon, '38), the results obtained are probably applicable to both forms.

#### RESULTS AND DISCUSSION

A tabulation of the data (table 1) shows a rather wide range of variation in requirement of  $\alpha$ -tocopherol. Within reasonable limits, the magnitude of the individual dose fed is

<sup>1</sup>This product was generously supplied by Hoffman-La Roche, Inc., Nutley, New Jersey.

TABLE I  
*a-tocopherol requirement.*

RABBIT NO.	SEX	TOTAL AMOUNT OF <i>a</i> -TOCOPHEROL FED	PERIOD OF PROTECTION	<i>a</i> -TOCOPHEROL REQUIREMENT	RABBIT NO.	SEX	TOTAL AMOUNT OF <i>a</i> -TOCOPHEROL FED	PERIOD OF PROTECTION	<i>a</i> -TOCOPHEROL REQUIREMENT
		mg.	days	mg./kg./day			mg.	days	mg./kg./day
Diet 113X									
598	M	40	114	0.16	678	F	7	15	0.30
599	M	40	114	0.16	625	F	13	26	0.31
543	M	5.8	28	0.19	629 <sub>1</sub>	M	20	32	0.31
654	M	10	26	0.19	659	M	8.1	22	0.32
634	F	3.2	16	0.20	588 <sub>1</sub>	M	38.4	60	0.34
650	M	10.6	25	0.23	628	M	25	44	0.34
595 <sub>1</sub>	F	36	72	0.23	658	F	9.4	23	0.35
595 <sub>2</sub>	F	23.5	36	0.23	629 <sub>2</sub>	M	26.5	31	0.36
630 <sub>1</sub>	F	22.5	53	0.23	663	M	10	18	0.36
588 <sub>2</sub>	M	27.5	48	0.24	600 <sub>1</sub>	F	40	58	0.37
637	F	14.8	36	0.24	672	F	5	8	0.37
594	M	42	87	0.25	676	M	7.5	17	0.37
602 <sub>1</sub>	F	42	73	0.26	601 <sub>2</sub>	M	23.8	32	0.39
602 <sub>2</sub>	F	30	40	0.26	635	F	15.5	34	0.40
603 <sub>2</sub>	M	30	42	0.26	661	F	12	23	0.40
593 <sub>2</sub>	M	21.2	32	0.27	593 <sub>1</sub>	M	45	61	0.41
589 <sub>1</sub>	F	45	75	0.28	601 <sub>1</sub>	M	40.8	65	0.43
603 <sub>1</sub>	M	42	73	0.29	662	M	26	40	0.44
620	M	5	13	0.29	664	F	20.3	39	0.44
589 <sub>2</sub>	F	29	35	0.30	585	M	38.4	59	0.45
600 <sub>2</sub>	F	24.1	35	0.30	671	F	5	9	0.49
614	M	33.6	54	0.30	590	M	42	53	0.53
630 <sub>2</sub>	F	26.3	42	0.30	586	F	61	75	0.60
Diet 13									
520 <sub>2</sub>	F	22	38	0.17	511 <sub>1</sub>	F	111	72	0.57
502 <sub>2</sub>	M	44	74	0.19	502 <sub>1</sub>	M	117	86	0.58
506 <sub>2</sub>	F	16	26	0.23	524	F	106	67	0.59
518	M	57.5	88	0.23	525	M	90	59	0.59
511 <sub>2</sub>	F	68	85	0.23	512 <sub>1</sub>	M	126	76	0.60
511 <sub>2</sub>	F	25	20	0.38	501 <sub>1</sub>	M	120	87	0.68
512 <sub>2</sub>	M	81	67	0.38	517	M	204	91	0.70
549 <sub>2</sub>	M	35.5	37	0.39	508	M	208	105	0.83
501 <sub>2</sub>	M	72	59	0.43	515	F	135	68	0.89
506 <sub>2</sub>	F	60	42	0.45	503	F	120	64	0.96
513	M	78	63	0.50	520 <sub>1</sub>	F	222	85	1.03
549 <sub>1</sub>	M	40	48	0.50	506 <sub>1</sub>	F	184	79	1.07

The subscripts <sub>1</sub>, <sub>2</sub>, <sub>3</sub> refer to the first, second and third test performed on the same rabbit.

Diet 113X. Standard deviation, S =  $\pm 0.095$ ; mean =  $0.32 \pm 0.037$ .

Diet 13 (first twelve animals). Standard deviation, S =  $\pm 0.12$ ; mean =  $0.34 \pm 0.11$ .

apparently of no importance, but the data reveal a direct relationship between the total amount of tocopherol fed and the requirement in milligrams per kilogram of body weight per day. Thus the requirements on diet 13 range all the way from 0.17 mg./kg./day to 1.07 mg./kg./day, the lower values corresponding to the smaller total intake while the higher values are shown by rabbits receiving the much greater total amounts of  $\alpha$ -tocopherol.

In many animals we failed to bring about a cure regardless of the amount of tocopherol fed. This was due to the fact that in some cases the tocopherol feeding was started too late or that too little tocopherol was given when the animals were already too seriously injured by severe E-avitaminosis. In some instances the animals were purposely fed subminimal quantities. Nevertheless, in nearly two-thirds of the 112 experiments successful cures were effected.

If the total amounts of tocopherol administered are plotted against the time during which the animals have been protected against dystrophy, one notes the following interesting things. In the case of the rabbits on diet 13 or diet 113X which received very small or only moderate supplements of tocopherol a good linear relationship is seen between the two variables; but in the case of rabbits on diet 13, which were fed massive amounts of tocopherol, this relationship is no longer linear. The demarcation is not sharp but the change from the linear to the non-linear correlation occurs when the total amount of tocopherol is in excess of 60 mg. In other words, as the quantity of  $\alpha$ -tocopherol fed increases beyond a certain limit it becomes progressively less effective in the protection against dystrophy.

The inefficient utilization of  $\alpha$ -tocopherol on the high levels of intake can best be explained by assuming that the animal tissues soon become saturated with the vitamin, the excess being excreted. Or, if a greater rate of destruction occurs, this takes place only after saturation of the tissues is complete. Such an interpretation is borne out, for instance, by the three tests performed on rabbit 511. This animal on a

total intake of 111 mg. (3 mg. per day) shows a minimum requirement of 0.57 mg./kg./day, but in another test with a total intake of 48 mg. (4 mg. per day) this value is only 0.23 mg./kg./day. Yet, when it was given a single massive dose of 25 mg. an intermediate degree of utilization of 0.38 mg./kg./day was obtained. Had the rate of destruction been greater on the higher levels, then it would be expected that the single

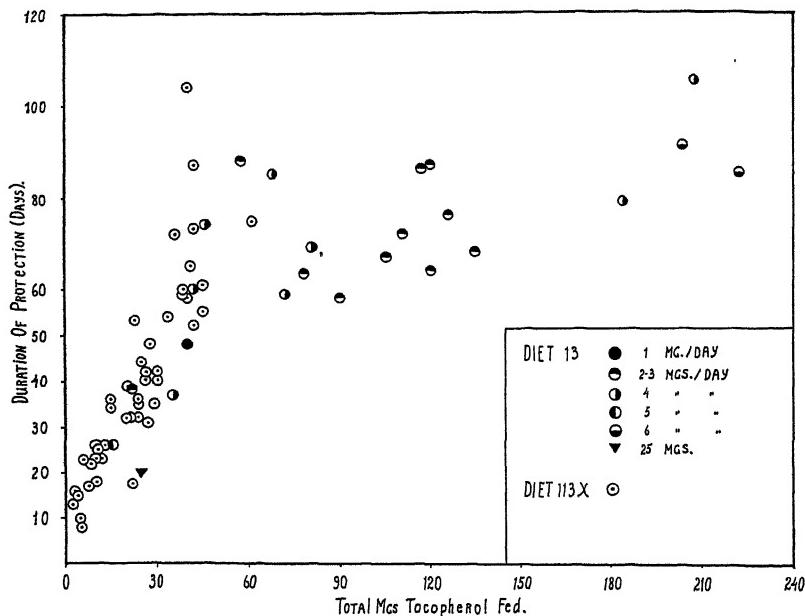


Fig. 1 Relation between the total amount of  $\alpha$ -tocopherol fed and the duration of protection against dystrophy.

massive dose would be very inefficient; but if the tissues, depleted of their vitamin E reserves, could store a large portion of this dose allowing the excess to "spill over", in this instance, about 40%, the result could be easily explained. Successive tests carried out with other animals also bear out this interpretation. Assuming that the increased vitamin E requirement of the animals on a high total intake of tocopherol means that the tissues had become saturated with the vitamin,

the excess being eliminated or otherwise destroyed, then one can make a rough estimate of the ability of the animals to store the vitamin. This would be measured by the duration of the interval from the time supplementation was discontinued until dystrophic symptoms reappeared in these rabbits. Of course, there still remain two uncontrollable variables, namely, the relative mass of musculature of individual animals and the length of time during which only suboptimal reserves of the vitamin are available to the animal. Nevertheless, even a rough estimate of this "storage" capacity may be of some value. For the twelve rabbits on diet 13 with minimum requirements of tocopherol of 0.57 mg./kg./day or more this time-measure of storage capacity varies uniformly from 19 to 57 days, the average being 36 days. From these considerations it is obvious that a safe and convenient method of E supplementation is to feed at one time a calculated amount sufficient to last 1, 2 or even 3 weeks.

The values obtained on diet 13 when compared with those for diet 113X within the same general level of total supplementation are on the whole somewhat higher. By the methods of statistical analysis<sup>2</sup> the difference between the two means is not significant. Furthermore, the fact that animals on either diet become dystrophic in the same length of time, coupled with the remarkably good agreement between the lower requirements calculated on both diets, would indicate that these differences are not due to a greater amount of residual E in diet 113X but to the fact that few animals were used on diet 13 at the lower levels of total amounts of tocopherol fed.

In all probability several factors are responsible for the great range of variation in the minimum requirements, such as intestinal destruction of vitamin E, variations in the ability

<sup>2</sup>The formulae used as a test for significance and for determining the probable error are those derived from the theory of fiducial limits. The interpretation of these values for the probable error is the same as for those obtained by the usual methods, but the concept of fiducial limits is theoretically sounder than that of the "probable error". For a brief discussion of this, see J. F. Kenney, Mathematics of Statistics, vol. II, pp. 177-186, 1939, D. Van Nostrand Co.

to absorb it, as well as the variable error in determining the period of protection. Leaving out of consideration the high values obtained on diet 13 where massive quantities were fed, one can see from the histograms (fig. 2) that most of the animals manifest an apparent need for between 0.2–0.4 mg. of dl- $\alpha$ -tocopherol per kilogram of body weight per day. The mean for diet 113X is  $0.32, \pm 0.037$ , and for the twelve animals on diet 13, excluding the high values associated with massive E intake, the average is  $0.34 \pm 0.11$ .

These values are considerably lower than the approximation of 1 mg./kg./day given by Mackenzie and McCollum ('40).

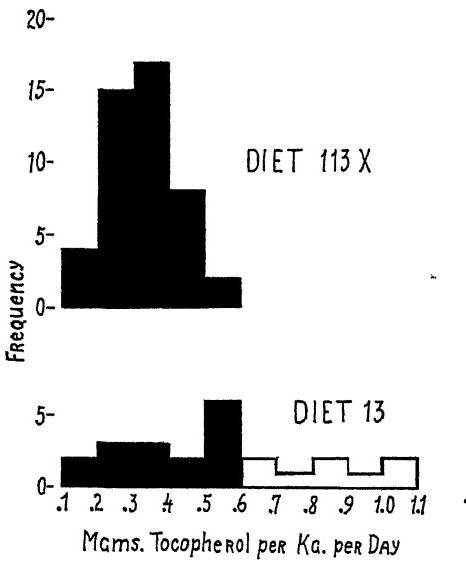


Fig. 2 Frequency distribution of tocopherol requirements in mg./kg. body weight/day. The unshaded portion represents the values when excessive amounts of vitamin were fed.

#### *Parenteral use of tocopherol*

To eliminate the variable factors of intestinal destruction and absorption, a number of experiments were made with intramuscular injections of dl- $\alpha$ -tocopheryl acetate. According to Knowlton, Hines and Brinkhous ('39) rats apparently

utilize the injected ester and it would seem reasonable to suppose that rabbits do also. Mattill ('40) reported that injections of the acetate ester were very inefficient in curing dystrophic rabbits. In our experience, too, twelve rabbits injected with from 5 to 10 mg. failed to be cured. Only a single animal, given one injection of 20 mg. in 1 ml. of olive oil, recovered.

Mattill ('40) suggested that possibly the ester to be utilized must first be hydrolyzed, the necessary enzyme being present only in the intestine. An alternative interpretation is that the oil is so poorly absorbed that the animals succumb before an adequate concentration of vitamin E in the tissues can be effected. To test this view four animals were injected with 5-10 mg. of tocopherol ester (in 1 ml. of oil) in four or five different places. The effect of such multiple injections should be the same as of a single injection of 20-25 mg. in 1 ml. of oil. A fifth animal was similarly treated with the free natural tocopherol.<sup>3</sup> All five animals so treated died. One rabbit treated with the ester apparently improved at first but then suffered a relapse. In order to clarify this problem experiments are now in progress with a water soluble tocopheryl ester.

#### *Sex factor*

No difference in tocopherol requirements could be detected between male and female rabbits, nor was there any difference in the per cent of cures effected in either sex. Mature animals of both sexes also showed the same vitamin E requirement.

#### *Age factor*

It was considered possible that the requirement for tocopherol would change with maturity. To test this, cured animals which had already become mature were made dystrophic again and retested, but no difference in E requirements due to age could be detected. If in the first period the animal had been given a large total amount, the E requirement for that period was, of course, higher. But whenever the total intake

<sup>3</sup>Kindly furnished by Merck and Co., Inc., Rahway, New Jersey.

of tocopherol was below approximately 60 mg., no significant change in E requirements could be found on comparing the immature animal with the mature.

#### SUMMARY

1. The minimum requirement of dl- $\alpha$ -tocopheryl acetate for rabbits as determined by the cure of dystrophy is probably about 0.32 mg. per kilogram of body weight per day (calculated as a free tocopherol).
2. The interval of time for which a supplement cured and protected the animal against dystrophy was directly proportional to the total amount of tocopherol fed when this amount was below approximately 60 mg. The reasons for this behavior are discussed.
3. The results of the present experiment suggest a convenient method for administering vitamin E supplements.
4. Sex or age do not affect the vitamin E requirements.
5. Intramuscular injections of dl- $\alpha$ -tocopheryl acetate failed to cure dystrophy.

#### LITERATURE CITED

- EPPSTEIN, S. H., AND S. MORGULIS 1940 Minimum daily requirement of rabbits for  $\alpha$ -tocopherol. Proc. Soc. Exp. Biol. and Med., vol. 45, p. 715.
- EVANS, H. M., AND G. A. EMERSON 1939 Degrees of sterility in the female rat held on E-free rations. Science, vol. 89, p. 438.
- EVANS, H. M., G. A. EMERSON AND O. H. EMERSON 1939 Preservation of the seminiferous epithelium and of fertility in male rats by prophylactic administration of alpha-tocopherol. Am. J. Physiol., vol. 126, p. 487.
- GOETTSCH, M., AND A. M. PAPPENHEIMER 1931 Nutritional muscular dystrophy in the guinea pig and rabbit. J. Exp. Med., vol. 54, p. 145.
- KARRER, P., H. FRITZSCHE, B. H. RINGIER AND H. SALOMON 1938 Synthese des  $\alpha$ -Tocopherols. Helvet. Chim. acta, vol. 21, p. 820.
- KNOWLTON, G. C., H. M. HINES AND K. M. BRINKHOUS 1939 Cure and prevention of vitamin E-deficient muscular dystrophy with synthetic  $\alpha$ -tocopherol acetate. Proc. Soc. Exp. Biol. and Med., vol. 42, p. 804.
- MACKENZIE, C. G., AND E. V. MCCOLLUM 1940 The cure of nutritional muscular dystrophy in the rabbit by alpha tocopherol and its effect on creatine metabolism. J. Nutrition, vol. 19, p. 345.
- MATTILL, H. A. 1940 Muscular dystrophy in rabbits and the autoxidation of animal fats. J. Nutrition, vol. 19, p. 13. (Am. Inst. of Nutrition Proc.) and private communication.

## THE RIBOFLAVIN CONTENT OF FISH PRODUCTS

F. L. BILLINGS, JACOB BIELY, HERBERT FISHER  
AND CARL HEDREEN

*The University of British Columbia and Canadian Fishing Company,  
Vancouver, Canada*

(Received for publication June 9, 1941)

The distribution of riboflavin in meat and meat products intended for human consumption has been reported by Darby and Day ('38), as determined by the rat growth method, and more extensively by Mickelsen, Waisman and Elvehjem ('39), as determined by the microbiological method. Hodson ('40) studied, by means of the fluorometric method, the influence of dietary riboflavin on the amount of this vitamin found in various tissues of chickens.

The riboflavin potency of livers from various domestic animals has been studied rather extensively. Daniel and Munsell ('37) and Hoagland and Snider ('30) determined biologically the riboflavin content of beef and pork liver. Additional data on the liver from the cow, calf, sheep, lamb and hog were reported by Saffry et al. ('40). Mickelsen et al. ('39) found that the riboflavin content of beef liver varied between 105 and 125 µg. per gram of dried material; veal liver between 100 and 135; pork liver between 80 and 90; and that one sample of lamb liver contained 90 µg. Hodson ('40) found that the liver of chickens fed a low riboflavin diet contained from 35 to 46 µg. of riboflavin per gram of dry tissue, while the liver of chickens fed a high riboflavin diet contained 94 to 129 µg. per gram of dry tissue. The results obtained by these investigators are in agreement, in that they have all shown that liver tissue is one of the most potent sources of riboflavin.

In view of the high riboflavin content of liver tissue obtained from domestic animals, it seemed of importance to determine the riboflavin content of fish livers and other by-products.

The riboflavin content of some of the more common poultry feedstuffs has recently been determined microbiologically by Culton and Bird ('41) and Billings and Biely ('40). On the whole, good agreement has been found between the biological and the microbiological values (Pratt et al., '40; Emmett et al., '41). The accuracy of the microbiological method *per se* has been determined for a large number of poultry feed samples by Billings and Biely ('40) in this laboratory and in co-operation with other laboratories.

#### MATERIALS AND METHODS

The results of the microbiological assay of fifteen liver meals, twelve by-product meals and ten fish meals for riboflavin are summarized in table 1. Livers from thirteen species of fish (samples 1 to 15, table 1) and several by-products were gathered during the autumn of 1940 and the winter of 1940-1941. The livers were collected into 40-pound tinned cans, sealed, and stored under refrigeration at an average temperature of -20°C.

Five-pound samples of the frozen livers were thawed and dried under vacuum at a temperature of 50-60°C. The greater portion of the liver oil was removed from the dried material by pressure using a Carver laboratory press. All the dried meals were analyzed for moisture and ether extract. The same procedure was followed with all samples of liver included in the study.

The viscera and roe meals, 16 to 23, were prepared in semi-commercial quantities (200 to 300 pounds) by drying in vacuo in a steam-jacketed dryer at 50-60°C. Meals from salmon milt and herring muscle, 24 and 26, were prepared in the laboratory, while meals from salmon heads, 25, were prepared in semi-commercial quantities.

"Stick water" meal was prepared by evaporating "stick water" under vacuum and drying it into a meal. "Stick

TABLE 1  
*The riboflavin content of fish products*

SAMPLE NO.	SPECIES OF FISH	TISSUE	SOURCE	MOIS-TURE	FAT	RIBOFLAVIN CONTENT	
						Meal	Moisture and fat-free meal
1	Red Cod	Liver	Laboratory	7.8	29.9	39.0	62.6
2	Red Cod	Liver	Laboratory	7.3	32.5	40.0	66.4
3	Ling Cod	Liver	Laboratory	4.1	23.9	48.0	66.6
4	Alaska Black Cod	Liver	Laboratory	16.5	16.7	60.0	89.8
5	Grey Cod	Liver	Laboratory	13.3	29.8	57.0	100.1
6	Chum Salmon	Liver	Laboratory	8.2	9.3	43.0	52.1
7	Pink Salmon	Liver	Laboratory	29.2	13.1	56.0	97.4
8	Sockeye Salmon	Liver	Laboratory	29.6	22.5	38.0	79.3
9	White Spring Salmon	Liver	Laboratory	8.0	21.4	62.5	88.5
10	Souffin Shark	Liver	Laboratory	15.4	18.3	64.0	96.5
11	Dog Fish	Liver	Laboratory	11.5	31.6	48.0	84.3
12	Halibut	Liver	Laboratory	17.7	18.9	44.0	69.4
13	Albacore Tuna	Liver	Laboratory	8.2	30.7	62.0	101.4
14	Herring	Liver	Laboratory	8.5	16.4	67.0	89.2
15	Sockeye Salmon	Liver	Laboratory	9.8	17.1	67.5	92.3
16	Alaska Black Cod	Viscera <sup>1</sup>	Laboratory	15.5	26.3	30.5	52.4
17	Chum Salmon	Viscera <sup>1</sup>	Semicommercial	12.4	9.7	33.0	42.3
18	Pink Salmon	Viscera <sup>1</sup>	Semicommercial	8.7	13.1	33.5	42.8
19	Sockeye Salmon	Viscera <sup>1</sup>	Semicommercial	12.5	14.8	40.5	55.7
20	Salmon	Roe	Semicommercial	10.3	4.8	22.7	26.7
21	Salmon (heat treated)	Roe	Semicommercial	12.5	18.2	24.5	35.3
22	Salmon (raw)	Roe	Semicommercial	13.2	11.6	35.7	47.4
23	Salmon	Roe	Semicommercial	8.9	8.8	34.2	41.5
24	White Spring Salmon	Milt	Laboratory	9.0	3.9	10.0	11.4
25	Salmon	Heads	Semicommercial	9.7	20.5	15.0	21.4
26	Herring	Muscle	Laboratory	3.5	23.7	10.0	13.7
27	"Stick meal"	Stick	Semicommercial	3.1	22.1	25.0	33.4
28	Pilchard	Meal	Commercial	....	....	13.2	
29	Pilchard	Meal	Commercial	....	....	11.8	
30	Pilchard (oily)	Meal	Commercial	....	....	13.6	
31	Salmon	Meal	Commercial	....	....	9.0	
32	Herring	Meal	Commercial	....	....	9.2	
33	Herring (oily)	Meal	Commercial	....	....	11.0	
34	Herring (vacuum dried)	Meal	Commercial	....	....	16.0	
35	Herring (Japanese) sun-dried	Scrap	Commercial	....	....	18.0	
36	Herring (Japanese) sun-dried	Scrap	Commercial	....	....	22.0	
37	Herring (Japanese) sun-dried	Scrap	Commercial	....	....	22.0	

<sup>1</sup> In the process of cleaning salmon probably two-thirds of the liver stays with the head, since part of the liver is in that portion of the stomach cavity which is cut off with the head. The pink and chum viscera meal contains that portion of the liver which remained in the body cavity, but roe was removed. The sockeye viscera meal was prepared from material, however, from which both roe and all remaining liver was removed.

water" is ordinarily a waste product of the wet process of producing fish meal (Beall, '33). It is the watery solution remaining after separation of oil from the liquid pressed from cooked fish. It contains much of the water-soluble vitamins present in the original fish (Lassen, '40).

Samples 28 to 35 are typical of fish meals manufactured and used in British Columbia. Samples 35 to 37 (herring scrap) represent a Japanese product ordinarily known to the trade as "sun-dried herring scrap."

Representative samples of each of the meals were thoroughly ground and stored in glass bottles in a dark room. The procedure followed in the microbiological tests was similar to that described by Snell and Strong ('39) and Billings and Biely ('40). Each sample of meal was tested at several levels, and appropriate controls (positive and negative) were used in each instance. In addition to the microbiological test, one sample of liver meal, viscera meal and roe meal was tested biologically on chicks. Close agreement was obtained between the biological and microbiological tests (Pratt, Biely and Fisher, '40).

#### DISCUSSION

It will be seen from the data presented in table 1 that with one or two exceptions the riboflavin content of the fish liver meals is uniformly high. The meals prepared from the fish viscera were found to rank second to fish livers in riboflavin content. Fish muscle meal, as is the case with the muscle of domestic animals, was found to be low in riboflavin as compared with liver meal. It is not without interest to note that while fish roe has been found to contain appreciable amounts of riboflavin, herring milt is definitely low. "Stick water" meal was found to contain appreciable amounts of riboflavin. While it is thus a fairly valuable source of riboflavin, it should be remembered that "stick water" meal protein has a low biological value (Pratt, Biely and Fisher, '40).

Fish meals, as will be seen from table 1, vary considerably in riboflavin content. Wilgus et al. ('35) have shown that the

riboflavin content of fish meals may be affected by the method of manufacture and by the kinds of material used. Since liver meal and viscera meal are relatively high in riboflavin, the amount of these products included in fish meals would affect their riboflavin content.

The results of the microbiological determinations show that of the various fish products assayed for riboflavin, liver tissue is one of the most potent sources of this vitamin. It may be of interest to note that the distribution and relative content of riboflavin in the various tissues of sea fish are similar to those found in corresponding tissues of domestic animals. Our results are in agreement with those obtained by Lunde in Norway ('39), who found that fish liver and roe are especially rich in riboflavin.

#### SUMMARY

The riboflavin content of fish products has been determined according to the microbiological method of Snell and Strong.

Of the materials examined, fish liver meal proved to be richest in riboflavin content; viscera, roe and "stick water" meal were next in value; muscle meal and milt meal were lowest.

Fish meals showed considerable variation in their riboflavin content. This may be attributed to different methods of manufacture and various types of material used.

Fish liver meal, like liver meal prepared from domestic animals, is one of the richest natural sources of riboflavin.

#### LITERATURE CITED

- BEALL, DESMOND 1933 Loss in the effluent of pilchard reduction plants in British Columbia. Bul. No. 35, Biological Board of Canada, Ottawa, Canada.
- BILLINGS, F. L., AND JACOB BIELY 1940 The riboflavin content of poultry feeds. (Unpublished data.)
- CULTON, THOS. G., AND H. R. BIRD 1941 The riboflavin content of poultry feedstuffs. Poultry Science, vol. 20, p. 3.
- DANIEL, E. P., AND H. E. MUNSELL 1937 Vitamin content of foods. U. S. D. A. Misc. Pub. No. 275, p. 73.
- DARBY, W. J., AND P. L. DAY 1938 Riboflavin content of meats. J. Nutrition, vol. 16, p. 209.

- EMMETT, A. D., O. D. BIRD, R. A. BROWN, GAIL PEACOCK AND J. M. VANDENBELT  
1941 Determination of vitamin B<sub>2</sub> (riboflavin). Ind. Eng. and Chem.,  
vol. 13, p. 219.
- HOAGLAND, R., AND G. G. SNIDER 1930 Vitamin G in certain meats and meat  
by-products. J. Agr. Research, vol. 41, p. 205.
- HODSON, A. Z. 1940 The influence of dietary riboflavin on the content of this  
vitamin in chicken tissue. J. Nutrition, vol. 20, p. 377.
- LASSEN, S. H. 1940 Process of concentrating vitamins from fish press water.  
U. S. Patent no. 2188008, January 23, 1940.
- LUNDE, G. 1939 Neure Forschungen über die Vitamine in Fischen und Fisch-  
produkten Angewandte Chemie, vol. 52, p. 521.
- MICKELSEN, OLAF, H. A. WAISMAN AND C. A. ELVEHJEM 1939 The distribution  
of riboflavin in meat and meat products. J. Nutrition, vol. 18, p. 517.
- PRATT, J. M., JACOB BIELY AND HERBERT FISHER 1940 The nutritive value of  
B. C. fish meals. (Unpublished data.)
- SAFFRY, OLGA B., H. S. COX, B. L. KUNERTH AND M. M. KRAMER 1940 A.  
Biological assay of riboflavin in the liver of the cow, calf, sheep, lamb  
and hog. J. Nutrition, vol. 20, p. 169.
- SNELL, E. E., AND F. M. STRONG 1939 A. Microbiological assay for riboflavin.  
Ind. Eng. and Chem., vol. 11, p. 346.
- WILGUS, H. S., JR., L. C. NORRIS AND G. F. HEUSER 1935 Haddock meal:  
Effect of manufacturing process upon nutritive value. Ind. and Eng.  
Chem., vol. 27, p. 419.

# THE EFFECT OF AUTOCLAVING ON THE NUTRITIVE VALUE OF THE PROTEINS IN COTTONSEED MEAL<sup>1</sup>

H. S. OLCOTT<sup>2</sup> AND T. D. FONTAINE<sup>3</sup>  
*Mellon Institute, Pittsburgh, Pennsylvania*

TWO FIGURES

(Received for publication May 7, 1941)

The literature describing the effect of heat on the food value of proteins has been summarized by Hayward, Steenbock and Bohstedt ('36), Waisman and Elvehjem ('38), and Greaves, Morgan and Loveen ('38). Casein, edestin, and meat proteins are adversely affected while soy and other bean proteins are improved by such treatment. Osborne and Mendel ('17) and Gallup ('28) reported that excessive steaming or autoclaving of cottonseeds or commercial cottonseed meals damaged the protein. However, interpretation of their data is complicated by the presence of the toxic substance, gossypol, or derivatives resulting from it by various treatments. The problem is sufficiently important to warrant further investigation inasmuch as most of the 2,000,000 tons of cottonseed cake and meal produced annually in this country is subjected to a cooking procedure prior to the pressing operation. It is then used as a source of protein in stock feeds. Information relating to the effect of cooking on the nutritive value of the proteins might be helpful in modifying the procedure to improve the

<sup>1</sup> Contribution No. XXVI from the Multiple Fellowship of the Cotton Research Foundation.

<sup>2</sup> Present address: Western Regional Research Laboratory, Albany, California.

<sup>3</sup> Present address: Southern Regional Research Laboratory, New Orleans, La.

food value of cottonseed meals. In the present study, gossypol-free cottonseed meats, prepared by ethyl ether extraction, were used to determine the effect of autoclaving on the nutritive value of the proteins.

#### EXPERIMENTAL

Whole cottonseed meats<sup>4</sup> were ground to pass a 20-mesh screen and extracted with ethyl ether in a large scale Soxhlet-type extractor capable of holding 15 pounds of meal in one charge. After drying, the oil- and gossypol-free meats were re-ground (burr-mill) to pass a 60-mesh screen. Portions were treated in a steam-jacketed autoclave by spreading in thin layers ( $\frac{1}{2}$ -1 cm.) on shallow trays. The temperature of the steam was measured by a thermometer set into an outlet through which a slight flow of steam was allowed to escape. The temperature of autoclaving remained constant at 123-124°C. (17 pounds) and the period of exposure to such treatment was measured from the time the inner chamber had reached 120°C.

The various cottonseed preparations were evaluated by their ability to stimulate growth in ad libitum feeding. Male rats (Sprague-Dawley) at weaning were placed in individual screen-bottom cages. They were weighed thrice weekly and food consumption data were recorded. The equicaloric diets had the compositions shown in table 1, with the vitamin-B complex furnished by liver extract and thiamine chloride as suggested by Elvehjem.<sup>5</sup>

The results of feeding the meal autoclaved for different periods of time and at 12 and 24% protein levels are presented in figure 1 and table 2. The conclusions have been based upon the results obtained at the 12% protein level. The groups of rats fed the meals at the 24% protein level, although few in number, have been included because they furnished a good check upon the other results, and because the better rate of growth, characteristic of experience with varying amounts of

<sup>4</sup>Courtesy of W. H. Jasspon, Perkins Oil Co., Memphis, Tennessee.

<sup>5</sup>Private communication from C. A. Elvehjem, University of Wisconsin.

other proteins (reviewed by Hamilton, '39), showed that no traces of gossypol or other toxic substances remained in the meal.

The nutritive value of the cottonseed meal decreased during autoclaving until, after 2 hours of such treatment, it was capable only of maintaining weight when fed at the 12% protein level. Although the varied conditions used by different investigators prevent direct comparison, the cottonseed

TABLE 1  
*Composition of diets*

Ethyl ether-extracted cottonseed meats	24.8 <sup>1</sup>	49.6 <sup>1</sup>
Hydrogenated cottonseed oil <sup>2</sup>	19.5	19.0
Sucrose	51.7	27.4
Salts <sup>3</sup>	2	2
Liver extract <sup>4</sup>	2	2
Haliver oil <sup>5</sup>	20 mg. (1 drop) per 100 gm.	
Protein content (N × 6.18)	12.7	25.0
In addition, each animal received 50 µg. of thiamine chloride in solution by mouth thrice weekly.		

<sup>1</sup> Approximate composition also of the diets containing extracted meats autoclaved for  $\frac{1}{2}$ , 1, and 2 hours.

<sup>2</sup> Crisco. The amount used was calculated to bring the total lipid concentration to 20%, approximately.

<sup>3</sup> Hubbell, Mendel and Wakeman ('37) mixture no. 351.

<sup>4</sup> Liver concentrate, 1: 20. Contains nitrogen equivalent to 50% protein. Purchased from the Wilson Laboratories Co.

<sup>5</sup> According to manufacturer's assay, contained 50,000 vitamin A and 10,000 vitamin D units (U.S.P. XI) per gram.

proteins appear to be somewhat more sensitive to heat damage than other proteins. Thus, Waisman and Elvehjem ('38) autoclaved edestin at 120°C. for 5 hours and found that the rat growth increment (one pair) decreased from 105 to 25 gm. (18% protein, 6 weeks growth period). Seegers and Mattill ('35) heated dried beef liver at 120° for 72 hours and found a marked decrease in protein value. Greaves, Morgan and Loveen ('38) reported that the nutritive value of casein, expressed in terms of grams of gain per gram of protein eaten, declined from 2.4 to 1.4 after heating the casein 24 hours in an

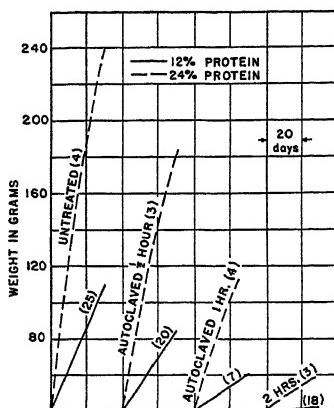


Fig. 1 Male rat growth rates obtained with diets containing ether-extracted cottonseed meats autoclaved at 17 pounds pressure for different lengths of time. The figures in parentheses indicate the number of animals for which the curves are an average.

oven at 130° (12% protein level). Mitchell, Burroughs and Beadles ('36) observed a slight decrease in biological value when peanuts were roasted at 204–232° for 30 to 35 minutes. The difference between the effect of wet heat and dry heat on the proteins obviously requires further investigation. In the case of soy bean proteins tested at an 18% level, autoclaving for 1½ hours at 125° increased the nutritive value from

TABLE 2  
The effect of autoclaving on the nutritive value of the proteins in ether-extracted cottonseed in ad libitum feeding.

TREATMENT OF MEAL	PROTEIN CONTENT OF DIET	NO. RATS	DAYS	GROWTH GAINS		AVERAGE FOOD CONSUMPTION	GM. OF GAIN PER GM. OF PROTEIN CONSUMED
				Range	Average		
None	12.7	7	31	66–72	68.0	268	1.99
Autoclaved ½ hr.	12.5	8	31	42–54	45.0	227	1.58
Autoclaved 1 hr.	12.5	4	31	13–20	17.5	137	1.08
Autoclaved 2 hr.	12.9	3	31	2–4	3.3	100	0.28
None	25.0	3	33	174–179	176.7	383	1.84
Autoclaved ½ hr.	24.95	3	31	108–175	142.7	311	1.84
Autoclaved 1 hr.	24.7	4	31	82–92	84.0	260	1.31
Autoclaved 2 hr.	24.7	3	35	19–30	23.0	179	0.52

0.3 to 1.2 gm. of gain per gram protein eaten, whereas dry heat was much less effective (Hayward, Steenbock and Bohstedt, '36).

#### AMINO ACID SUPPLEMENTATION STUDIES

In an attempt to determine which amino acids were destroyed or rendered biologically unavailable by the steam treatment, experiments were undertaken in which crystalline amino acids were used to supplement diets containing the 2-hour autoclaved ether-extracted meal fed at a 12% protein level. During a 15-day preliminary period, five rats were given the basal diet (A), five were given diet A in which 1% glycine replaced 1% sugar (B), and five were given diet A in which 2% glycine replaced 2% sugar (C). Glycine was used in order to ascertain the effect of adding a non-essential amino acid. No growth differences were detectable. One rat from each group was then placed upon a basal diet in which a portion of the sugar was replaced by supplements corresponding to 2% lysine (D), 1% lysine (E), 1% lysine and 1% histidine (F), 1% histidine (G), and 2% histidine (H). During the succeeding 15 days, the weight changes shown in figure 2 were observed. The animals receiving lysine gained an average of 11.5 gm., whereas those given histidine gained only 5.3 gm. Inasmuch as the lysine plus histidine inclusion produced no greater response than did lysine alone, it was concluded that the effect of histidine was negligible. This was confirmed in a subsequent 16-day experiment in which the growth of three rats given the basal diet plus histidine (1%) was equal to that of three others receiving glycine (1%).

The responses given by the two groups receiving 1 and 2% lysine indicated that the lower level was sufficient to overcome the lack of this amino acid in the autoclaved meal.

At the end of the experimental periods, several of the animals were transferred to diets containing untreated cottonseed meal. They immediately began to grow at rates of 3 to 4 gm. a day indicating that the metabolic responses to the proper amino acid combination had not been impaired.

Waisman and Elvehjem ('38) reported that lysine was the limiting factor in autoclaved edestin and Greaves, Morgan and Loveen ('38) concluded that lysine was the first amino acid damaged in heated casein, and histidine the second. Harris and Mattill ('40) showed that a loss in free amino nitrogen paralleled a loss in in vitro digestibility of meat proteins after

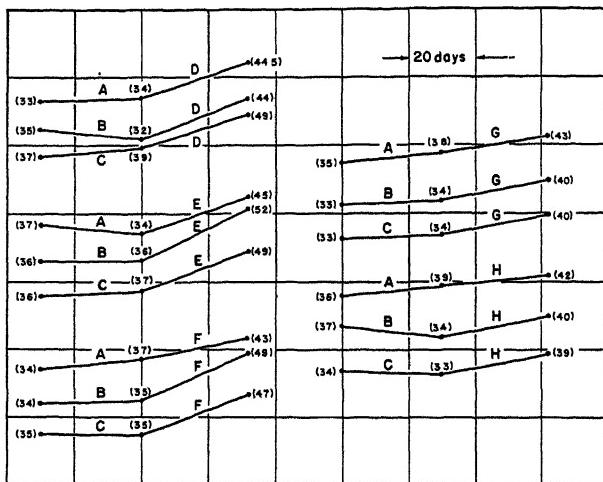


Fig. 2 Rat weight changes observed with amino acid supplementation of the ration containing ether extracted cottonseed meats autoclaved for 2 hours (12% protein level). A, no supplement; B, 1% glycine; C, 2% glycine; D, 2% lysine (3% lysine dihydrochloride); E, 1% lysine (1.5% lysine dihydrochloride); F, 1% lysine and 1% histidine (1.5% lysine dihydrochloride and 1.25% histidine hydrochloride); G, 1% histidine (1.25% histidine hydrochloride); and H, 2% histidine (2.5% histidine hydrochloride). In each case the added compounds replaced an equivalent amount of sugar. The figures in parentheses represent the rat weights in grams.

hot alcohol extraction. In the present instance, the lack of marked response to lysine and histidine would appear to indicate that either these amino acids were available in adequate or almost adequate amounts in the autoclaved cottonseed meal, or the unavailability of one or more other amino acids was sufficiently critical to mask the benefit of the addition of lysine or histidine.

## SUMMARY

As measured in ad libitum growth experiment with rats, steam autoclaving (17 pounds pressure) markedly reduced the nutritive value of the proteins in gossypol-free (ether-extracted) cottonseed meats. The 2 hour-autoclaved meal when present in quantity sufficient to give 12% protein in the diet permitted a gain in grams per gram protein eaten of 0.52 compared with 2.0 for the unautoclaved meal. Animals fed the 2 hour-autoclaved meal were only slightly benefitted by supplementation with lysine. Supplementation with histidine was ineffective.

## LITERATURE CITED

- GALLUP, W. D. 1928 The digestibility of the proteins of some cottonseed products. *J. Biol. Chem.*, vol. 76, p. 43.
- GREAVES, E. O., A. F. MORGAN AND M. K. LOVEEN 1938 The effect of amino-acid supplements and of variations in temperature and duration of heating upon the biological value of heated casein. *J. Nutrition*, vol. 16, p. 115.
- HAMILTON, T. S. 1939 The growth, activity, and composition of rats fed diets balanced and unbalanced with respect to protein. *J. Nutrition*, vol. 17, p. 565.
- HARRIS, R. L., AND H. A. MATTILL 1940 The effect of hot alcohol on purified animal proteins. *J. Biol. Chem.*, vol. 132, p. 477.
- HAYWARD, J. W., H. STEENBOCK AND G. BOHSTEDT 1936 The effect of heat as used in the extraction of soy bean oil upon the nutritive value of the protein of soy bean oil meal. *J. Nutrition*, vol. 11, p. 219.
- HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. *J. Nutrition*, vol. 14, p. 273.
- MITCHELL, H. H., W. BURROUGHS AND J. R. BEADLES 1936 The significance and accuracy of biological values of proteins computed from nitrogen metabolism data. *J. Nutrition*, vol. 11, p. 257.
- OSBORNE, T. B., AND L. B. MENDEL 1917 The use of cottonseed as food. *J. Biol. Chem.*, vol. 29, p. 289.
- SEEVERS, W. H., AND H. A. MATTILL 1935 The effect of heat and hot alcohol on liver proteins. *J. Biol. Chem.*, vol. 110, p. 531.
- WAISMAN, H. A., AND C. A. ELVEHIJEM 1938 The effect of autoclaving on the nutritive value of edestin. *J. Nutrition*, vol. 16, p. 103.



# DIFFERENCES IN THE BEHAVIOR OF RATS AND MICE TOWARDS DEFICIENCIES OF CERTAIN MEMBERS OF THE VITAMIN B COMPLEX<sup>1</sup>

JOHN R. FOY AND LEOPOLD R. CERECEDO

*Department of Chemistry, Fordham University, New York*

FIVE FIGURES

(Received for publication June 18, 1941)

At least two well-recognized types of dermatitis have been observed in rats fed on diets deficient in different fractions of the vitamin B complex. One of these is the specific dermatitis or acrodynia due to vitamin B<sub>6</sub> deficiency and the other is the non-specific dermatitis due to pantothenic acid deficiency. In the present work we have sought to duplicate these skin manifestations in mice.

On diets usually employed to produce acrodynia in the rat, that is, a vitamin B complex-free diet supplemented with thiamine and riboflavin, mice show only a non-specific dermatitis which does not respond to vitamin B<sub>6</sub> but is completely cured by the further addition of pantothenic acid. Uncomplicated vitamin B<sub>6</sub> deficiency can be produced in both rats and mice by supplementing a vitamin B complex-free diet with thiamine, riboflavin, and some source of the "filtrate factor" freed from vitamin B<sub>6</sub>. However, the characteristic acrodynia seen in rats under these conditions does not appear in mice, not even on low-fat diets.<sup>2</sup>

<sup>1</sup> Presented at the meeting of the American Chemical Society at St. Louis, April, 1941.

<sup>2</sup> The reduction in the amount of fat in the diet minimizes the possible curative effect of the unsaturated fatty acids on the acrodynia and emphasizes the difference between the symptoms in rats and mice.

## EXPERIMENTAL

In the experiments with mice, weanling albino animals from a strain reared in our laboratory were used in all cases. They were placed on the basal diets at 21 to 25 days of age, at which time they weighed from 8 to 12 gm. In the rat experiments, animals of the Wistar strain were used. They were also 21 to 25 days of age and weighed 35 to 40 gm. at weaning. The percentage composition of the diets used is given in table 1.

TABLE 1  
*Percentage composition of the diets.<sup>1</sup>*

CONSTITUENTS	DIET			
	A	B	C	D
"Vitamin-free" casein <sup>2</sup>	31	30		
Ether-extracted casein			30	20
Sucrose	38	55	63	75
Osborne and Mendel salt mixture	7	7	7	5
Hydrogenated cottonseed oil <sup>3</sup>	21	5		
Cod liver oil		3	3	

<sup>1</sup> Vitamin supplements were used as indicated in the text.

<sup>2</sup> Labco.

<sup>3</sup> Crisco.

Diets C and D were supplemented with concentrates of vitamins A and D.<sup>3</sup> The concentrates were dissolved in ether and evaporated on casein so that at least 6000 I.U. of vitamin A and 600 I.U. of vitamin D were present in each 100-gm. portion of the diet. When used for feeding and injections the water soluble vitamins (thiamine,<sup>4</sup> riboflavin,<sup>4</sup> nicotinic acid,<sup>4</sup> pyridoxine,<sup>5</sup> and pantothenic acid<sup>6</sup>) were dissolved in 0.1 N HCl and kept in the icebox. The solutions were neutralized before use. When they were included in the diet an aqueous solution of the vitamins was evaporated on a small

<sup>3</sup> Generously supplied by Dr. I. Harris.

<sup>4</sup> Obtained from Merck and Co., Rahway, N. J.

<sup>5</sup> First made available to us through the courtesy of Dr. Samuel Lepkovsky and later obtained from Merck and Co.

<sup>6</sup> Generously supplied by Merck and Co.

amount of casein which was then thoroughly mixed with the other constituents.

*Experiments on mice receiving the vitamin B complex-free diet plus limited supplements of the crystalline factors*

After the discovery of vitamin B<sub>6</sub> deficiency in the rat had been reported by György ('34, '35), it was decided to repeat this work on mice under similar conditions. The experiments were carried out on five groups of animals. The results are presented in figure 1, the legend of which gives the pertinent details.

Groups 1 to 4 inclusive (fig. 1) were fed diet A. Since a high fat content of the diet is known to exert a sparing action on certain factors of the vitamin B complex (Birch and

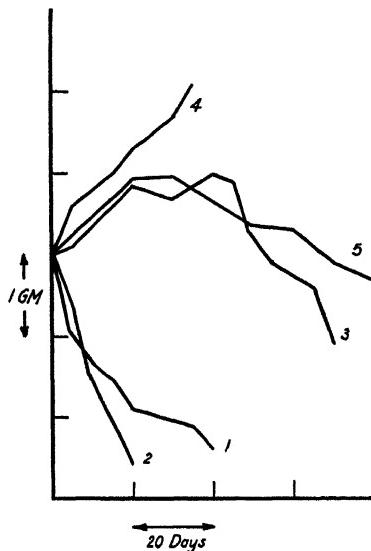


Fig. 1 Average growth curves of five groups of mice kept on a vitamin B complex-free diet supplemented with various factors of the vitamin B complex. Groups 1, 2, 3 and 4 received diet A and daily doses of the following supplements: Group 1 (twenty-one animals), 2 µg. thiamine and 5 µg. riboflavin (curve 1); group 2 (nine animals), 2 µg. thiamine (curve 2); group 3 (eight mice), 4 µg. thiamine, 5 µg. riboflavin and 10 µg. nicotinic acid (curve 3); group 4 (eight animals), 4 µg. thiamine and 10 µg. riboflavin (curve 4). Group 5 (forty mice) received diet B plus 5 µg. thiamine and 10 µg. riboflavin per day (curve 5).

György, '36; Stirn et al., '39), and since unsaturated rats in particular show a curative action on vitamin B<sub>6</sub> deficiency (Salmon, '40; Schneider et al., '40), the high-fat (21%) ration (diet A) was abandoned in favor of the low-fat (5%) mixture (diet B). Group 5, consisting of forty mice, received diet B supplemented with 5 µg. of thiamine and 10 µg. of riboflavin. After a slight initial growth these animals lost weight and died within 80 days (curve 5). As in the previous experiments, the symptoms associated with vitamin B<sub>6</sub> deficiency in the rat were not evident, but certain non-specific symptoms were observed. That these mice were not suffering primarily from vitamin B<sub>6</sub> deficiency is shown by the fact that the administration of as much as 20 µg. of pyridoxine daily for 10 to 20 days to each of ten animals after the dermatitis had developed was entirely without effect either on the symptoms or on the growth of the mice.

The following symptoms were seen to a greater or lesser degree in all animals that did not receive supplements of pantothenic acid or of the filtrate factor. The mice developed a humped back, awkward gait, and yellow matted fur which became thin, falling out in patches. Occasionally wet brownish scales appeared over most of the body, and in a few cases ulcerous sores were observed in the region of the axilla and groin. The animals were extremely emaciated, the skin being drawn tightly over the body so that the injection of the vitamin solution was made difficult. The extremely sensitive and irritable condition of the animals manifested itself in a characteristic kicking twitch of the hind legs.

From these results it is apparent that small amounts of thiamine and riboflavin, adequate when supplementing more complete diets, are definitely subminimal on the incomplete diets, and that under conditions in which the requirement for the vitamin B complex is only partially met, an increased amount of fat in the diet gives the animals a better chance for growth and health than the low-fat diets. Although these experiments indicate a difference between rats and mice, the diet used with mice was somewhat higher in protein and salts

than that commonly employed in rat experiments. It was therefore decided to observe the effect on the growth and symptoms of both rats and mice kept on the same diet when one vitamin B complex factor at a time was added to the basal ration.

*The influence of certain factors of the vitamin B complex on the growth and symptoms of rats and mice maintained on identical vitamin B complex-free rations*

Two groups of forty-eight mice and eighteen rats were each divided equally into six groups and given diet B with the supplements listed in table 2.

TABLE 2  
*Supplements furnished to the rats and mice in comparative studies on these two species.*

GROUP	SUPPLEMENTS (IN MICROGRAMS PER DAY)							
	Thiamine chloride		Riboflavin		Pyridoxine		Nicotinic acid	
	Mice	Rats	Mice	Rats	Mice	Rats	Mice	Rats
1								
2	5	20						
3	5	20	10	40				
4	5	20	10	40	20	20		
5	5	20	10	40			20	40
6	5	20	10	40	20	20	20	40

The crystalline vitamins were dissolved in 0.1 cc. of water and injected into the mice but fed to the rats. The growth curves for both the rats and the mice are given in figures 2 and 3, printed side by side to facilitate comparison.

*Group 1.* These animals received only the basal diet. Both the mice and rats lost weight and developed a humped back and an emaciated, unkempt appearance, but no specific symptoms were evident (curve 1).

*Group 2.* In this experiment diet B was supplemented with thiamine. The mice, after a slight initial growth, lost weight until death, while the rats just about maintained their weight.

Since the animals lived longer, the symptoms were more fully developed, but the characteristic acrodynia was not evident in either case (curve 2).

*Group 3.* In these animals which were given diet B plus thiamine and riboflavin, growth was better than that of the two preceding groups (curve 3). The symptoms observed in the mice were those that have already been described. The rats, however, developed symptoms characteristic of vitamin

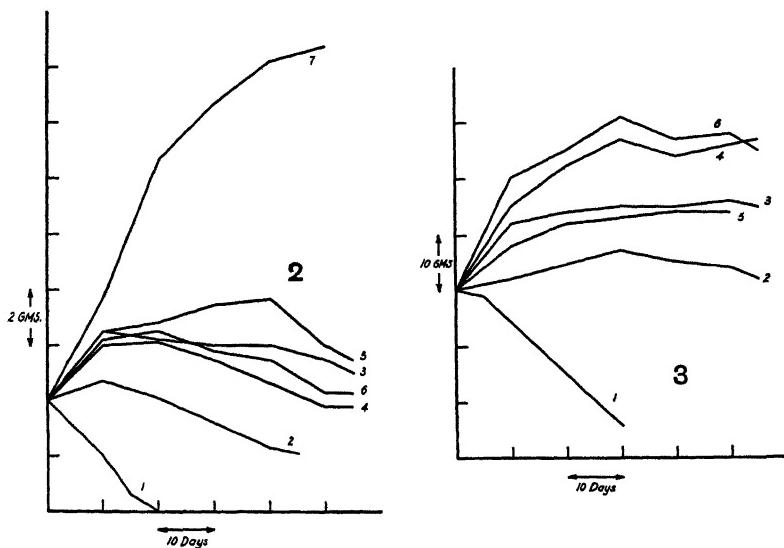


Fig. 2 Effect of various crystalline factors on the growth response of mice maintained on a vitamin B complex-free ration (diet B). Group 1 received only diet B (curve 1). The other six groups were given diet B and the following supplements (see table 2): Group 2, thiamine (curve 2); group 3, thiamine and riboflavin (curve 3); group 4, thiamine, riboflavin and pyridoxine (curve 4); group 5, thiamine, riboflavin and nicotinic acid (curve 5); group 6, thiamine, riboflavin, pyridoxine and nicotinic acid (curve 6); group 7, thiamine, riboflavin, pyridoxine and pantothenic acid. The animals in group 7 were not litter mates of the other mice in this experiment but the large difference in the growth rate is significant. In experiments in which the growth rates are similar small differences are significant only if litter mates are used in each group. Thus curve 7 cannot be compared with the normal growth shown in curve 4 of figure 4.

Fig. 3 Effect of various crystalline factors on the growth response of rats maintained on diet B. Group 1 received only diet B (curve 1). The other five groups (curves 2-6) received the same supplements which were given to the corresponding groups in the mouse experiment (fig. 2).

B<sub>6</sub> deficiency, that is, swollen paws and ears with cracking and denuding of the skin of the paws and muzzle.

It would appear from these results that on the same diet, mice do not develop the same symptoms as rats. Whereas the rat develops the characteristic acrodynia, the mouse develops a non-specific dermatitis of the type usually associated with filtrate factor deficiency in the rat.

*Group 4.* In this experiment the animals were given diet B plus thiamine, riboflavin, and pyridoxine. The growth of the mice did not differ significantly from that obtained with thiamine and riboflavin alone, while the rats showed a definite growth response to the added vitamin B<sub>6</sub> (curve 4). Except that the usual symptoms were preceded by an alopecia and a dry dandruff-like dermatitis, the syndrome in the case of the mice was that previously described. The rats, on the other hand, were free from the acrodynia seen in group 3, and developed instead the non-specific dermatitis attributed to filtrate factor deficiency and now known to be cured by pantothenic acid.

*Groups 5 and 6.* Those animals receiving nicotinic acid in addition to the other factors differed little in growth and symptoms from animals in the corresponding groups that did not receive this factor (curves 5 and 6).

For comparison we include the growth curve of a group of mice receiving diet B plus thiamine, riboflavin, pyridoxine, and pantothenic acid (Sandza and Cerecedo, '41). These animals grew at a rate that was about 80% of normal and were free from all symptoms (curve 7).

If we compare the results obtained with these two species, the most striking difference is the lack of response to vitamin B<sub>6</sub> and the similarity of the symptoms in all groups of mice, while in the rats two distinct syndromes are evident, and there is a definite growth response to each added factor except nicotinic acid. In the mouse, after the primary deficiency of thiamine and riboflavin has been satisfied, vitamin B<sub>6</sub> in the absence of pantothenic acid has little or no effect on growth or symptoms. A similar lack of response to concentrates of

factor W (prepared according to Black et al., '40) has been observed in mice subsisting on a diet containing only thiamine, riboflavin, and pyridoxine.<sup>7</sup> That the symptoms observed in the mice receiving these incomplete diets were caused by the absence of pantothenic acid is evident from the experiment of Sandza and Cerecedo ('41). A number of workers have reported this factor to be responsible for the cure of the non-specific dermatitis in rats.

*Attempts to produce uncomplicated vitamin B<sub>6</sub> deficiency in the mouse using liver filtrate as a source of the filtrate factor*

The necessity of feeding mice pantothenic acid or some source of the filtrate factor in order to produce uncomplicated vitamin B<sub>6</sub> deficiency, or to observe the effect of supplements

TABLE 3  
*Diets and supplements furnished to the mice and rats in the study of vitamin B<sub>6</sub> deficiency.*

GROUP	NO. OF		DIET	DAILY SUPPLEMENTS								
				Thiamine chloride		Riboflavin		Pyridoxine		Liver filtrate		
	Mice	Rats		μg.	Mice	Rats	μg.	Mice	Rats	cc.	Mice	Rats
1	3	3	B									
2	18	15	B	5	10	10	20			0.15	0.3	
3	6	10	B	5	10	10	20	5	10	0.15	0.3	
4	3	20	Stock <sup>1</sup>									

<sup>1</sup> The stock diet given to the mice had the following composition: Ground oats (Quick Quaker or Sunbeam) 50 parts, whole milk powder (Klim, or Merrell-Soule) 15 parts, and dried yeast (Northwestern) 10 parts. The rats received Purina Dog Chow as stock diet.

of pyridoxine, is evident from the above experiments. Consequently, a fuller's earth filtrate of autolyzed pig liver was prepared by the method of Lepkovsky and his co-workers ('36). Each cubic centimeter of this liver filtrate was equivalent to 10 gm. of whole liver. This experiment was conducted on four groups of mice and rats maintained on the diets and

<sup>7</sup> Unpublished data.

supplements listed in table 3. The growth curves for these animals are shown in figures 4 and 5.

*Group 1.* These animals received only diet B. The results with both the rats and the mice were the same as those that have been reported above (curve 1).

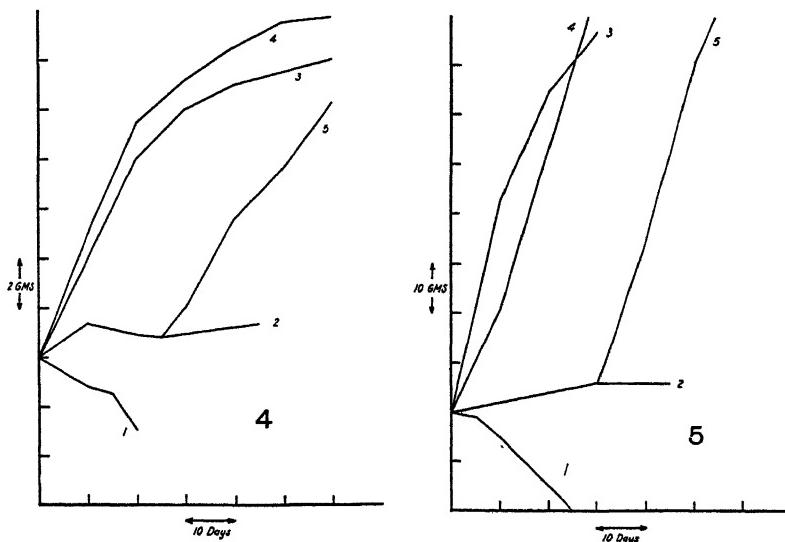


Fig. 4 Average growth curves of mice. In this experiment a source of the filtrate factor was given. Group 1 represents the negative controls (three animals) which were given only diet B (curve 1). Groups 2 and 3 received diet B and the following supplements: Group 2 (eighteen mice), thiamine, riboflavin and liver filtrate (curve 2); group 3 (six mice), thiamine, riboflavin, pyridoxine and liver filtrate (curve 3). Group 4 (three animals), was kept on the stock diet (curve 4). Some of the mice in group 2 received pyridoxine in addition to the other supplements (curve 5).

Fig. 5 Average growth curves of rats. This experiment is a duplicate of that conducted on mice and shown in figure 4. The number of animals in each group is given in table 3.

*Group 2.* The animals in this group received diet B plus thiamine, riboflavin and the liver filtrate. Both species maintained their weight within certain narrow limits (curve 2). No specific syndrome was evident in the mice though they became weak and emaciated, developed a humped back and walked with an awkward gait. In a few cases the eyes were

closed by a serous exudate. The rats developed the acrodynia characteristic of vitamin B<sub>6</sub> deficiency except for the absence of any pronounced dermatitis of the ears. Both the mice and the rats responded well to doses of vitamin B<sub>6</sub> in growth and cure of symptoms (curve 5).

*Group 3.* - In this experiment the animals received diet B plus thiamine, riboflavin, pyridoxine and the liver filtrate. In both the rats and the mice growth was good as compared to the normal growth obtained with the stock diet, and both were free from any gross symptoms (curve 3).

*Group 4.* These animals were given the stock diet (curve 4).

A fuller's earth filtrate of a 92% alcohol soluble liver extract,<sup>8</sup> was prepared by the method of Lepkovsky et al. ('36). Each cubic centimeter of this liver filtrate was equivalent to 10 gm. of whole liver. The experiment reported in the foregoing section (group 3) was repeated with a second group of thirty mice under the same conditions, except that the filtrate factor was that prepared from the 92% alcohol liver extract. The results with the mice as well as parallel experiments with rats were substantially the same.

It appears from these experiments that mice, as well as rats, require vitamin B<sub>6</sub> for normal nutrition, but that the symptoms of a deficiency of this factor do not express themselves in the same manner in each species, at least under the conditions of the present work.

Mice have been kept on diet B supplemented with thiamine, riboflavin, pyridoxine, and the liver filtrate for periods of 100 days. Though the growth was not quite normal, the animals weighed 85% as much as their litter mate controls kept on the stock diet, and showed no gross symptoms at any time. This is not in agreement with the findings of Norris and Hauschmidt ('40). On a diet not qualitatively different from our diet B, they observed failure of growth and skin lesions in their mice similar to the dermatitis which we observed in animals given only thiamine, riboflavin, and pyridoxine. However, in our experiments an equivalent of 1.5 gm. of liver was

<sup>8</sup> Supplied by the Wilson Laboratories, Chicago.

given per day while Norris and Hauschmidt gave an equivalent of only 0.5 gm. every other day. It has also been our experience that some preparations of liver filtrate do not furnish the activity expected, either in rats or mice. Norris and Hauschmidt do not report that they checked their results on rats, using the same source of liver filtrate.

In a recent report Martin ('41) has shown that the deficiency described by Norris and Hauschmidt is due to pantothenic acid. This is a confirmation of both our experiments in which the liver filtrate furnished the pantothenic acid, and those of Sandza and Cerecedo ('41) in which pantothenic acid was fed as such.

*Attempts to produce vitamin B<sub>6</sub> deficiency in the mouse,  
using a low-fat diet*

Since it had been shown that the dermatitis associated with vitamin B<sub>6</sub> deficiency may be cured by the presence of certain fatty acids in the diet, an attempt was made to produce the characteristic acrodynia in mice on a low-fat diet. These experiments were conducted on two groups of rats and mice.

*Group 1.* Forty mice were given diet C (table 1) supplemented with 2.5 mg. of thiamine, 5.0 mg. of riboflavin, and an equivalent of 750 gm. of liver as liver filtrate, per kilo of diet. Although the animals remained practically stationary in weight and became increasingly worse in appearance, no evidence of acrodynia was observed. The animals died before the symptoms of fatty acid deficiency could develop. Since the work of Burr and Burr ('29, '30) had been repeated on mice in this laboratory,<sup>9</sup> these symptoms were known to us.

*Group 2.* Twelve rats were given diet D (table 1) supplemented with 10 µg. of thiamine chloride, 20 µg. of riboflavin and an equivalent of 3 gm. of liver as liver filtrate per day. All animals developed the characteristic acrodynia of vitamin B<sub>6</sub> deficiency.

Thus it appears that even on low-fat diets mice, unlike rats, do not develop the specific dermatitis usually associated with a lack of vitamin B<sub>6</sub>.

<sup>9</sup> Unpublished data.

## SUMMARY

On vitamin B complex-free diets supplemented with thiamine and riboflavin, mice, unlike rats, do not develop a specific dermatitis nor do they exhibit any growth response to the addition of pyridoxine to their daily ration.

Mice fed a vitamin B complex-free diet develop a non-specific dermatitis in the presence of any or all of the known factors of the vitamin B complex (thiamine, riboflavin, pyridoxine, and nicotinic acid) with the exception of pantothenic acid.

On a vitamin B complex-free diet supplemented with thiamine, riboflavin, pyridoxine, and the filtrate factor, mice grow at a rate that is about 85% of normal and show no gross lesions of a deficiency of any kind over a period of 100 days.

## LITERATURE CITED

- BIRCH, T. W., AND P. GYÖRGY 1936 A study of the chemical nature of vitamin B<sub>6</sub> and methods for its preparation in a concentrated state. *Biochem. J.*, vol. 30, p. 304.
- BLACK, S., D. V. FROST AND C. A. ELVEHJEM 1940 The relation of vitamin B<sub>6</sub> and pantothenic acid to factor W studies. *J. Biol. Chem.*, vol. 182, p. 65.
- BURR, G. O., AND M. M. BURR 1929 A new deficiency disease produced by rigid exclusion of fat from the diet. *J. Biol. Chem.*, vol. 82, p. 345.
- 1930 On the nature and rôle of the fatty acids essential in nutrition. *J. Biol. Chem.*, vol. 86, p. 587.
- GYÖRGY, P. 1934 Vitamin B<sub>2</sub> and the pellagra-like dermatitis in rats. *Nature*, vol. 133, p. 498.
- 1935 Investigations on the vitamin B<sub>2</sub> complex: I. The differentiation of lactoflavin and the "rat anti-pellagra" factor. *Biochem. J.*, vol. 29, p. 741.
- LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. *J. Biol. Chem.*, vol. 115, p. 557.
- MARTIN, G. J. 1941 The mouse antialopecia factor. *Science*, vol. 93, p. 422.
- NORRIS, E. R., AND J. HAUSCHILDT 1940 A new factor of the vitamin B complex required by the albino mouse. *Science*, vol. 92, p. 316.
- SALMON, W. D. 1940 The supplementary relationship of vitamin B<sub>6</sub> and unsaturated fatty acids. *J. Biol. Chem.*, vol. 133, p. lxxxiii.
- SANDZA, J. G., AND L. R. CERECEDO 1941 Requirement of the mouse for pantothenic acid and for a new factor of the vitamin B complex. *J. Nutrition*, vol. 21, p. 609.
- SCHNEIDER, H., H. STEENBOCK AND B. R. PLATZ 1940 Essential fatty acids, vitamin B<sub>6</sub>, and other factors in the cure of rat acrodynia. *J. Biol. Chem.*, vol. 132, p. 539.
- STIRN, F. E., A. ARNOLD AND C. A. ELVEHJEM 1939 The relation of dietary fat to the thiamin requirements of growing rats. *J. Nutrition*, vol. 17, p. 485.

# THE ASSIMILATION OF FLUORINE BY RATS FROM NATURAL AND SYNTHETIC CRYOLITE AND FROM CRYOLITE-SPRAYED FRUITS<sup>1,2</sup>

MARGARET LAWRENZ AND H. H. MITCHELL

*Division of Animal Nutrition, University of Illinois, Urbana*

(Received for publication June 30, 1941)

In preceding papers from this laboratory, the effects of various dietary factors and methods and forms of administration on the assimilation of fluorine by growing rats have been studied. This report will be concerned with the effects of factors more specifically related to the fluorine in spray residues on fruits.

Cryolite is the most important fluorine-containing insecticide employed in the spraying of fruits (Carter and Busbey, '39). There are two forms of cryolite on the market at the present time, one the natural mineral and the other a synthetic product. While the former is more widely used as an insecticide, especially in the Northwest, the latter has been the form used in our experiments up to the present. There are reasons for believing that the two commercial cryolites may possess different assimilabilities and toxicities. The natural product is a purer form of sodium fluoaluminate,  $\text{Na}_3\text{AlF}_6$ , than is the domestic synthetic product (Carter, '39), and according to our own analyses it contains more fluorine,

<sup>1</sup> This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America and the Pennsylvania Salt Manufacturing Company.

<sup>2</sup> This investigation was conducted under the general supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the Director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

and its fluorine is somewhat less soluble. It seemed important, therefore, to compare the two products as to their assimilation by the growing rat.

The toxicity of cryolite for the insects infesting fruit against which it is effective is greater, within limits, the more finely the cryolite is ground. It seemed important to ascertain whether the fineness of division was a factor in determining the toxicity of cryolite for a higher form of animal life such as the rat.

During the weathering of sprayed apples and peaches on the tree, there is a possibility that the chemical composition of the spray residue may be changed, either through chemical reactions or by the differential leaching of more soluble constituents in the case of an impure chemical such as cryolite. These possible changes, although they have not, to the writers' knowledge, been investigated in the case of cryolite, may alter the assimilability of the contained fluorine. Hence, this possibility seemed worthy of study.

During the growth of the apple and during storage subsequent to harvesting, a waxy coating accumulates on the surface of the fruit (Markley and Sando, '31), the nature of which has been largely revealed by the investigations of Sando ('23, '31) and van der Haar ('24). The production of this wax coat apparently interferes with the commercial removal of spray residues from apples, and also its rate of formation may be stimulated by the application of sprays (Markley and Sando, '33). Therefore, it seemed expedient to study the possibility that waxing of the apple may impair the assimilation by animals of the fluorine component of the residue from cryolite-sprays.

#### PLAN OF THE EXPERIMENTS

The experiments were planned along the same general lines as those previously reported (for example, Lawrenz, Mitchell and Ruth, '39). In each of the four experiments to be reported, twelve pairs or trios of rats, depending upon the number of rations to be compared, were selected on the basis of sex,

litter membership and body weight, and were fed the experimental rations in equal amounts within the pair or trio. At the beginning of each experiment, samples of one to three or four rats were taken from each litter contributing rats to the experiment, for determination of the initial content of fluorine. After the consumption of a definite amount of food, generally 500 gm. or 1000 gm., unless unanticipated circumstances dictated otherwise in the case of individual pairs or trios, the experimental rats were sacrificed, their empty weights and body lengths determined, the carcasses separated into bones, teeth and soft tissues after autoclaving at 13 pounds pressure for 15 to 25 minutes depending upon the size of rat, and these three portions weighed and analyzed for fluorine by methods previously described (Lawrenz, Mitchell and Ruth, '39). During the feeding period, the rats were weighed weekly, and after the sixth week the teeth were examined weekly for the appearance of striations, using a jeweler's lens with a magnification of 4 X.

All experimental rations contained low levels of fluorine, approximating 10 p.p.m., in order that the results obtained with them may possess more significance with reference to the prevailing fluorine hazard in the diet of the American people. They were all designed to contain all of the known essential nutrients in adequate amounts.

In the first experiment a comparison was made of the assimilation of fluorine from a domestic synthetic cryolite<sup>3</sup> and from a natural Greenland cryolite.<sup>4</sup> The former was very finely divided, its particles being mostly 1  $\mu$  or less in diameter. The latter was obtained in two forms; one, the commercial product, consisted of much larger particles (5  $\mu$  or less) than those in the synthetic cryolite; the other, a specially prepared product, was ground to a degree of fineness comparable to that of the synthetic product. The fluorine contents of these three cryolite preparations were 44.9% for the synthetic cryolite, 46.2% for the coarse natural cryolite and 50.5% for

<sup>3</sup> Provided by the Aluminum Company of America.

<sup>4</sup> Provided by the Pennsylvania Salt Manufacturing Company.

the special fine natural cryolite. The higher fluorine content of the finely ground as compared with the coarsely ground natural cryolite may have been due to the variation among samples of the mineral or to the rejection, in the process of grinding, of coarser material relatively poor in fluorine. These three cryolite samples were added separately to basal rations of natural foods (wheat, dry milk solids), supplemented with minerals and cod liver oil, to form three experimental rations containing 9.6 p.p.m. of fluorine of which 8 parts were derived from the added cryolites. A comparison of the assimilation of fluorine from the ration containing the synthetic cryolite and from that containing the fine natural cryolite was expected to throw light upon any differential toxicity of the two preparations due to differences in chemical composition. A comparison of the two rations containing the finely ground and the coarsely ground natural cryolite should reveal any appreciable differential toxicity due to differences in particle size.

The second experiment was planned to test the assimilation of fluorine by the rat from unweathered and weathered natural cryolite in one comparison, and from weathered coarse natural cryolite and weathered fine natural cryolite in another. Through the kindness of Professor W. P. Flint, arrangements were made to secure, from a commercial orchard near Carbon-dale, Illinois, Elberta peaches that had been sprayed (rather late in the season) with a spray mixture containing in one case the coarsely ground natural cryolite, and in the other, the finely ground special natural cryolite. In addition, unsprayed peaches were secured from the same source. Under the direction of Dr. W. A. Ruth, these peaches were dried at a low temperature ( $70^{\circ}$  F.) and ground to a fine powder. The peach powders were found to contain the following amounts of fluorine: coarse cryolite sprayed, 100 p.p.m., fine cryolite sprayed, 94 p.p.m., and unsprayed, 1.5 p.p.m. The two sprayed-peach powders were incorporated into similar basal-rations containing equal amounts of casein, starch, sugar, and corn oil, with mineral and vitamin additions, in such propor-

tions as to provide 8 p.p.m. of fluorine. This required 8.5% of the fine-cryolite sprayed peach powder and 8.07% of the coarse-cryolite sprayed peach powder. To the latter ration was added enough of the unsprayed peach powder (0.43%) to equalize the content of peach powder in the two rations. A third ration was prepared by adding to the same mixture of basic foods 8.5% of unsprayed peach powder and enough finely ground natural cryolite to provide 8 p.p.m. of fluorine. Thus, the three rations to be tested in this experiment contained equal amounts of all organic and mineral nutrients including peach powder, and equal amounts of added fluorine in the form of unweathered fine natural cryolite, weathered fine natural cryolite, and weathered coarse natural cryolite. The total fluorine content of all rations was 9.6 p.p.m.

The third experiment was similar to the second except that weathered synthetic cryolite on sprayed Ben Davis apples, dried and ground, containing 10 p.p.m. of fluorine, was compared with unweathered synthetic cryolite mixed with unsprayed apple powder, containing 1.05 p.p.m. of fluorine, prepared from Huntsman apples. Both rations contained 65% of apple powder, equal and adequate amounts of casein, minerals, vitamins and other organic nutrients, and equal proportions of fluorine, i.e., 6.8 p.p.m.

The fourth experiment was concerned with a possible effect of the formation of a wax coat on sprayed apples on the assimilation of fluorine in spray residues. For this purpose, Jonathan apples sprayed with synthetic cryolite were used. One lot of freshly picked apples, not over-ripe, was dried immediately at a low temperature and ground to a fine powder under the supervision of Dr. Ruth. Another lot of the same apples was allowed to stand at a fairly warm temperature for several weeks to "wax up." They were then dried and ground. The apple powders contained 32 and 30 p.p.m. of fluorine, respectively, and were incorporated in the usual basal balanced mixture of organic and mineral ingredients in amounts to provide about 11 p.p.m. of fluorine. The content of apple powder in both rations was equalized at 35%

by adding a small amount (2.3%) of unsprayed and unwaxed apple powder to the ration containing the apple powder (unwaxed and sprayed) with the larger content of fluorine. Both rations contained, by actual analysis, 13.2 p.p.m. of fluorine.

#### RESULTS OF THE EXPERIMENTS

The average results of the four experiments are summarized in table 1. In table 2 will be found a statistical analysis of the paired data secured in the first experiment, using the method of Student ('25).

In the first experiment, the growth of the rats was quite similar on the three experimental rations and was fairly rapid, averaging 1.65 gm. per day. Tooth striations made their appearance in the various rats within a period extending from the end of the eighth week to the end of the ninth week, with no perceptible differences between groups.

The fluorine retention data for the first two series of rats, receiving the synthetic and natural cryolite ground to a comparable degree of fineness, showed highly significant differences, with reference to the fluorine content of bones and teeth and the total fluorine retention. An average of 13.1% more fluorine was retained by the rats receiving synthetic cryolite than by their trio mates receiving natural cryolite and the probability that this, or a greater, difference would occur as the result of uncontrolled fortuitous factors is only 0.013. The percentage difference in bone fluorine is 12.4 and in tooth fluorine, 5.1. Although the fluorine in the soft tissues, expressed either as milligrams or parts per million, averaged higher for the rats receiving synthetic cryolite than for those receiving natural cryolite, the probability of a chance outcome was much too large to be disregarded.

It may be concluded, therefore, that the fluorine in synthetic cryolite is definitely and significantly more completely assimilable by the rat than the fluorine of natural cryolite. Furthermore, this difference cannot definitely be attributed to a difference in size of particles, since the two cryolites were

TABLE I  
The growth data and fluorine metabolism data secured in the four experimental comparisons. Each figure is an average for twelve rats.

	EXPERIMENT 1			EXPERIMENT 2			EXPERIMENT 3			EXPERIMENT 4		
	Averages for rats receiving:			Averages for rats receiving:			Averages for rats receiving:			Averages for rats receiving:		
	Synthetic erythrite	Fine natural erythrite	Coarse natural erythrite		Unweathered natural erythrite	Weathered natural erythrite		Weathered synthetic erythrite	Unweathered synthetic erythrite		Unwaxed apples	Waxed apples
Initial body weight, gm.	39	39	38		49	49		49	50		50	33
Total gain in weight, gm.	183	183	185		207	204		149	157		136	137
Final body length, mm.	221	221	221		227	225		225	205		207	204
Total food consumed, gm.	1,000	1,000	1,000		1,000	1,000		1,000	884		500	500
Numbers of food refusals	13	7	12		13	8		9.6	12		8	8
Total intake of fluorine, mg.	9.65	9.63	9.70		9.6	9.6		9.6	6.002		6,600	6,600
Feeding period, days	111	111	111		118	118		118	90		69	69
Final empty body weight, gm.	222	222	223		256	253		254	199		169	169
Weight of dry, fat-free bones, gm.	11.487	11.723	11.381		11.896	11.911		11.666	10.253		10.319	8.485
Weight of soft tissues, gm.	0.408	0.427	0.422		0.462	0.449		0.459	0.409		0.399	0.360
Weight of teeth, gm.	199.6	197.9	201.3		234	231		232	179		187	154
Fluorine in bones, p.p.m.	313	272	265		291	295		298	251		252	305
Fluorine in bones, mg.	3.565	3.170	3.051		3.437	3.501		3.454	2.537		2.563	302
Fluorine in teeth, p.p.m.	180	163	163		143	156		153	208		216	176
Fluorine in teeth, mg.	0.073	0.070	0.068		0.066	0.070		0.070	0.085		0.086	0.067
Fluorine in soft tissues, p.p.m.	0.39	0.36	0.40		0.34	0.39		0.38	0.73		0.89	0.55
Fluorine in soft tissues, mg.	0.079	0.072	0.080		0.072	0.082		0.082	0.128		0.173	0.084
Total fluorine in carcass, mg.	3.717	3.312	3.199		3.575	3.653		3.606	2.750		2.822	2.667
Initial fluorine content, mg.	0.230	0.228	0.227		0.318	0.317		0.316	0.317		0.320	0.214
Total fluorine retained, mg.	3.487	3.084	2.972		3.257	3.336		3.290	2.433		2.502	2.515
Total fluorine retained, per cent	36.14	32.03	30.70		33.92	34.75		34.27	41.83		42.47	38.12

\* Because the soft tissue sample of one of the rats in this series was obviously contaminated with fluorine, these averages involve eleven instead of twelve rats.

\* Omitting one sample (from a rat that received unweathered erythrite) with a suspiciously high F content, suggesting contamination with bone, this average becomes 0.141.

ground to comparable degrees of fineness. Determinations of the relative solubilities of the fluorine in the two cryolites offer a clue to their different physiological effects. Solubility tests over a limited period of time were made upon 5 mg. and 25 mg. samples of the two cryolites, using water, 0.2% HCl and 0.5% HCl as the solvents, the latter two solvents simulating the acidity in gastric contents and gastric juice, respectively. In both series of tests the fluorine in synthetic cryolite was

TABLE 2  
*Statistical analysis of fluorine data.*

	SYNTHETIC VS. NATURAL CRYOLITE				FINELY GROUNDED VS. COARSELY GROUNDED NATURAL CRYOLITE			
	Mean difference <sup>1</sup>	Standard deviations of differences	t value	P	Mean difference <sup>2</sup>	Standard deviations of differences	t value	P
Fluorine in bones, p.p.m.	+40.9	22.1	6.14	<0.00007	+7.7	15.5	1.65	0.064
Fluorine in bones, mg.	+0.395	0.294	4.45	0.0005	+0.130	0.173	2.49	0.015
Fluorine in teeth, p.p.m.	+17.1	11.6	4.88	0.0002		insignificant		
Fluorine in teeth, mg.	+0.004358	0.00463	2.56	0.013		insignificant		
Fluorine in soft tissues, p.p.m.		insignificant				insignificant		
Fluorine in soft tissues, mg.		insignificant				insignificant		
Total fluorine retained, mg.	+0.403	0.296	4.51	0.0005	+0.117	0.173	2.24	0.025

<sup>1</sup> The positive sign indicates that the mean difference favors the synthetic cryolite.

<sup>2</sup> The positive sign indicates that the mean difference favors the finely ground natural cryolite.

more readily soluble in water than the fluorine in natural cryolite, the difference amounting to 10 to 11%. In dilute HCl, the tests with the 5 mg. samples revealed no consistent difference in solubility while the tests with the larger samples revealed a 10% difference.

Comparison of the second and third series of rats, receiving natural cryolite of two degrees of fineness, reveals a definite and significant difference in total fluorine retention and especially in the fluorine content of the bones, averaging about 4%. The greater fluorine retentions were secured with the

finer ground cryolite, although solubility tests failed to reveal any consistent differences in the solubility of fluorine in the two mineral preparations, either in water or dilute HCl.

The results of experiment 2, also summarized in table 1, reveal no significant differences among the three series of rats on application of Student's method of assessing the significance of paired differences, either in rate of gain, appearance of tooth striations, fluorine contents of tissues or total fluorine retention. Thus, the effect of weathering on the assimilability of fluorine from finely ground natural cryolite is inappreciable, and the effect of grinding, shown to be a real effect in the preceding experiment, is obliterated by weathering. This result is analogous to the finding of Shields, Mitchell and Ruth ('39) that the weathering of lead arsenate spray residue on apples does not appreciably modify the assimilability of the contained lead.

In the test of the effect of weathering on synthetic cryolite, the tooth striations characteristic of fluorosis appeared at the beginning of the tenth week of feeding, and in nine of the twelve pairs appeared first among the rats on the unweathered cryolite. Three rats on the weathered cryolite showed no striations at the end of 16 weeks of feeding, when they were sacrificed for analysis. Although the fluorine contents of the bones, teeth and soft tissues, and the total fluorine retentions, averaged higher for the rats that received unweathered cryolite than for their pair mates that received weathered cryolite, the differences between averages were quite insignificant, when tested statistically, except, anomalously, in the case of the soft tissues. Here, leaving out the one suspiciously high analysis noted in the table, the average difference was 0.0126 mg., the standard deviation of differences 0.0143 mg., and the probability of a fortuitous event only 0.0076. Although the data of the experiment are not equally convincing in respect to each of the items measured, it seems reasonable to conclude that, with synthetic cryolite in contrast to natural cryolite, weathering on the fruit will in some degree lower the assimila-

lability of its fluorine, conceivably by leaching out some or all of the more soluble compounds of fluorine contained in it.

The fourth experiment revealed no great difference between the assimilation of fluorine from waxed and unwaxed apples. Although the average retention of fluorine during the feeding period and the total content of fluorine in bones and teeth were somewhat smaller for the rats receiving the waxed-apple powder, the differences between pair mates in any one comparison were not consistently in the direction indicated by the respective averages. An application of the statistical method of analysis revealed such a high probability that the outcome may have resulted from chance that any conclusion that waxing was the determining factor, or even an appreciable factor, could not be defended.

Reference to table 1 will show that the concentration of fluorine in the teeth of the rats receiving waxed-apple powder averaged considerably lower than the concentration of fluorine in the teeth of the rats that received unwaxed-apple powder, 176 p.p.m. as compared with 186 p.p.m. The differences between pair mates favored the ration containing unwaxed apples in nine of the twelve pairs and averaged 10.7 p.p.m. With a standard deviation of differences of 13.9 p.p.m. the probability of a fortuitous result is only 0.0135, so that the conclusion that it was brought about by the difference in wax coating on the apples supplying most of the dietary fluorine can be defended strongly. However, the total fluorine contents of the teeth, when submitted to a similar analysis, yield a probability of 0.12, surely too large to be neglected. Tooth striations appeared indiscriminately among the two groups of rats beginning with the seventh week of feeding, so that these observations afford no reason for distinguishing between the two experimental rations with reference to fluorine toxicity. It is possible, however, that the method of preparing the apples for test, particularly the fineness of grinding, may have largely obliterated an appreciable effect of waxing on the assimilation of fluorine residues when the apples are consumed in the intact form.

## SUMMARY AND CONCLUSIONS

Four experiments have been performed on growing rats, involving the controlled feeding and chemical analysis of twenty-four trios and twenty-four pairs of animals. These experiments were designed to detect and measure differences in retention by animals of the fluorine in natural Greenland and domestic synthetic cryolite and possible modification of such retentions by the particle size of mineral, by the weathering of spray chemicals on the fruit and by the development of the wax coating on the surface of sprayed apples.

The results obtained appear to warrant the following conclusions:

1. The fluorine of synthetic cryolite is appreciably more completely retained by animals than the fluorine of natural cryolite, probably a result of the greater solubility of the fluorine contained in it.
2. The degree of fineness to which cryolite is ground may modify the assimilation of the contained fluorine.
3. The weathering of natural Greenland cryolite on sprayed fruit does not appreciably affect its assimilability by animals nor presumably its toxicity.
4. On the other hand, with synthetic cryolite, weathering on the fruit will in some degree lower the assimilability of its fluorine, conceivably by leaching out some, or all, of the more soluble compounds of fluorine contained in it. Thus, the differences observed between synthetic and natural cryolite tested in an unweathered form will tend to disappear when the two cryolites are applied to fruit in spray mixtures and subjected to weather conditions.
5. Possibly the development of a wax coat on apples sprayed with cryolite impairs the assimilability of the contained fluorine, as it seems to render more difficult the removal of spray residue by commercial washing. The experiments reported in this paper relating to this subject, while not particularly assuring in support of this conclusion, quite probably did not permit the full effect of waxing to be exerted.

## LITERATURE CITED

- CARTER, R. H. 1939 Significant properties of some cryolite materials offered for insecticidal use. *J. Econ. Ent.*, vol. 32, pp. 490-492.
- CARTER, R. H., AND R. L. BUSBEY 1939 The use of fluorine compounds as insecticides, a review with annotated bibliography. U. S. Dept. Agr., Bureau of Ent. and Plant Quarantine, Div. of Insecticide Investigations, Washington, D.C., February, 1939, pp. 145.
- HAAR, A. W. VAN DER 1924 Untersuchungen über der Saponine und verwandte Körper. XIII. Die Identität von Malol mit Urson (Ursolsäure). *Rec. Trav. Chim. Pays-Bas.*, vol. 43, pp. 548-549.
- LAWRENZ, M., H. H. MITCHELL AND W. A. RUTH 1939 The comparative toxicity of fluorine in calcium fluoride and in cryolite. *J. Nutrition*, vol. 18, pp. 115-125.
- MARKLEY, K. S., AND C. E. SANDO 1931 Progressive changes in the waxlike coating on the surface of the apple during growth and storage. *J. Agr. Res.*, vol. 42, pp. 705-722.
- 1933 Possible changes in the waxlike coating of apples caused by certain spray and other treatments. *Plant Physiol.*, vol. 8, pp. 475-478.
- SANDO, C. E. 1923 Constituents of the wax-like coating on the surface of the apple. *J. Biol. Chem.*, vol. 56, pp. 457-468.
- 1931 Ursolic acid. *J. Biol. Chem.*, vol. 90, pp. 477-495.
- SHEIELDS, J. B., H. H. MITCHELL AND W. A. RUTH 1939 The effect of apple constituents on the retention by growing rats of lead contained in spray residues. *J. Nutrition*, vol. 18, pp. 87-97.
- "STUDENT" 1925 New tables for testing the significance of observations. *Metron*, vol. 5, pp. 105-120.

# $\alpha$ -TOCOPHEROL REQUIREMENT OF THE RAT FOR REPRODUCTION IN THE FEMALE AND PREVENTION OF MUSCULAR DYSTROPHY IN THE YOUNG

MARIANNE GOETTSCH AND ALWIN M. PAPPENHEIMER

*Departments of Biochemistry and Pathology, College of Physicians and Surgeons,  
Columbia University, New York City*

(Received for publication June 30, 1941)

Vitamin E is essential for normal reproduction in the female rat (Evans and Bishop, '23; Mattill and Stone, '23; Sure, '23-'24) and for the prevention of muscular dystrophy in the young (Evans and Burr, '28; Olcott, '38; Goettsch and Ritzmann, '39). Biological methods for estimating the vitamin are but roughly quantitative (Palmer, '37; Bacharach, '38), and relatively little has been recorded upon the  $\alpha$ -tocopherol requirement of the rat (Evans, Emerson and Emerson, '36; Karrer and Keller, '38; Bacharach, '38; Mason, '40).

This is a report on the potencies of natural  $\alpha$ -tocopherol, synthetic *dl*- $\alpha$ -tocopherol, and synthetic *dl*- $\alpha$ -tocopherol acetate with respect to:

*A. Cure of sterility in the female rat.* (1) Production of litter: (a) size; (b) number of living young. (2) Number of second and third litters. (3) Success of lactation: (a) number of young surviving 10 days; (b) incidence of muscular dystrophy.

*B. Prevention of muscular dystrophy in the young rat.*

## EXPERIMENTAL

### *Diets and supplements*

The diets (I and II), similar to those used by Evans and Burr ('27), had the following percentage composition:<sup>1</sup> casein<sup>2</sup> 29.1

<sup>1</sup> The figures for diet I are those which immediately follow the name of the ingredient; the figures for diet II are given in parentheses.

<sup>2</sup> Merck's technical.

(26.6); cornstarch, raw 36.3 (33.3); lard 20.0 (18.3); yeast, bakers' dried 9.0 (16.6); salt mixture (Hawk and Oser, '31) 3.6 (3.4); and cod liver oil<sup>3</sup> 2.0 (1.8). Diet II containing more yeast was used during the period of lactation. The diets were freshly prepared every week.

Three preparations of  $\alpha$ -tocopherol were employed: synthetic *dl*- $\alpha$ -tocopherol acetate<sup>4</sup> and natural and synthetic  $\alpha$ -tocopherols.<sup>5</sup> The tocopherols were dissolved in ethyl laurate or sesame oil. The solutions were prepared by weight in such proportions that for feeding, 0.5 mg. of  $\alpha$ -tocopherol was contained in 1 drop, and for injection the desired amount was present in 0.1 cc. of sesame oil. The solutions were stored at 4°C. and used within 1 week of preparation.

### Methods

Repeated tests were carried out with small groups of animals over a period of 2 years. This tended to cancel out variations due to unrecognized differences in food composition, possible seasonal influences, or differences in individual response.

Young female rats, which during lactation had been on a natural-foods diet containing small amounts of vitamin E (Mason and Bryan, '38), were reared on diet I from the time of weaning (21 days) and bred with normal males at 90 days (Evans and Bishop, '23; Palmer, '37). It was held unnecessary to prove the sterility of the rats by a preliminary resorption gestation, as in previous experiments forty-six females reared and bred on diet I had failed to cast a single litter.

Single doses of  $\alpha$ -tocopherol were given orally or parenterally on or before the fifth day following the finding of sperm in the vaginal smear. If implantation did not occur, as indicated by the absence of erythrocytes in the smear on the fourteenth day of pregnancy, the rat was bred at the following estrus. Failure of implantation after positive mating

<sup>3</sup> Mead Johnson.

<sup>4</sup> Ephynal, of Hoffman-La Roche, Inc., through the courtesy of Dr. F. Gudernatsch.

<sup>5</sup> Merck and Co., through the courtesy of Dr. J. M. Carlisle.

occurred in forty (10%) out of 404 gestations under these conditions.

If the first gestation resulted in the birth of living young, the female was given diet II and permitted to suckle them. Record was made of the number of young which survived the first 10 days of lactation, and from the seventeenth to twenty-fifth day the rats were observed daily for symptoms of muscle dystrophy. Survivors were killed on the twenty-fifth day. Autopsies were performed upon every rat in the litter and representative muscle specimens preserved for histological examination. The diagnosis of muscle lesions was based upon microscopic evidence.

After the period of lactation the females were given diet I and bred again in 2 weeks. The second gestation was studied in the same way as the first. If litters were born, the rat was bred for the third time. All rats were bred until the occurrence of a resorption gestation, evidence for which was based upon the following findings: sperm in the vaginal tract, followed on the fourteenth day of gestation by the "implantation sign"; from the nineteenth to twenty-third day, a loss in body weight of not more than 5 gm. daily. Since the presence of resorption sites in the uterus was not confirmed directly, the possibility exists that some of the so-called resorptions may have been pseudo-pregnancies. However, in the latter case (Evans, '28), the "placental sign" usually occurs on the tenth or eleventh day, 2 or 3 days before this sign is found in true pregnancies.

In tests for the prevention of muscular dystrophy, female rats that had been used in the anti-sterility test, or young rats reared on diet I, were kept on diet I and bred. During the first 5 days of gestation 1 gm. of fresh wheat germ oil was given by mouth. All of the young born were kept for lactation, during which period the diet of the mother was changed to diet II. On the fifteenth to seventeenth day of lactation some of the young were given orally or parenterally single doses of  $\alpha$ -tocopherol. The remaining young were kept as controls and were not given any supplementary feeding. During the rest

of the period of lactation, they were observed as described above. Litters in which the untreated controls did not develop muscle lesions were discarded.

### RESULTS

#### *Cure of sterility in the adult female rat*

**Fertility.** As proposed by Bacharach and Allchorne ('38), this was expressed as the per cent of gestations which resulted in the birth of young, regardless of litter size or number of living young; furthermore, a loss in body weight of 15 gm. or more during the twenty-second day was assumed to indicate the casting of a litter.

TABLE 1

*Prevention by  $\alpha$ -tocopherol of resorption during gestation in rats on an E-low diet.*

	$\alpha$ -TOCOPHEROL: SINGLE DOSE (IN MILLIGRAMS) AT THE BEGINNING OF THE FIRST GESTATION					
	0.5	1.0	2.5	10.0	20.0	50.0
Natural						
No. of gestations	3	14	8	4	3	—
No. of litters	0	11	8	3	3	—
Per cent fertility	0	79	100	75	100	—
Synthetic						
No. of gestations	3	14	5	4	3	2
No. of litters	0	8	5	4	3	2
Per cent fertility	0	57	100	100	100	100
Synthetic acetate						
No. of gestations	3	32	17	4	8	4
No. of litters	0	16	14	4	8	4
Per cent fertility	0	50	82	100	100	100

The fertility of females that subsisted on diet I and were fed single doses of  $\alpha$ -tocopherol at the beginning of the first gestation is shown in table 1. It will be seen that the three preparations gave essentially the same results: 0.5 mg. of  $\alpha$ -tocopherol was insufficient to permit the birth of a litter; 1.0 mg. induced litters in thirty-five (58%) of sixty rats and 2.5 mg. in twenty-seven (90%) of thirty rats; at 10, 20 and 50 mg., the incidence was nearly 100%. The biological variation was very great; for instance, among fifteen groups of

rats receiving 1.0 mg. of  $\alpha$ -tocopherol there were some that had no young, while in others, all of the rats cast litters.

The minimum amount of  $\alpha$ -tocopherol which permitted the birth of young to at least 85% of rats under the given experimental conditions was approximately 2.5 mg. This value lies within the limits, 1.0 to 3.0 mg., given for the minimum dose by Evans, Emerson and Emerson ('36), Karrer and Keller ('38), and Bacharach ('38).

TABLE 2

*Influence of  $\alpha$ -tocopherol in rats on an E-low diet upon litter size and the number of young born alive, surviving the early lactation period, and developing muscular dystrophy.*

	MILLIGRAMS OF $\alpha$ -TOCOPHEROL: SINGLE DOSE AT BEGINNING OF GESTATION				
	1.0	2.5	10.0	20.0	50.0
No. of litters	28	22	11	13	5
No. of young	194	190	97	111	43
Av. young per litter	6.9	8.6	8.8	8.5	8.6
No. living young	131	163	90	109	43
Per cent	67	86	93	99	100
No. surviving 10 days	61	95	22	78	29
Per cent	46	58	24	71	67
No. kept for muscular dystrophy study (not given $\alpha$ -tocopherol)	16	28	18	70	29
No. with muscular dystrophy	16	25	15	65	22
Per cent	100	89	83	93	76

*Litter size.* The litters varied in size from one to fourteen rats. It will be seen in table 2 that in rats receiving 1.0 mg. of  $\alpha$ -tocopherol the average number of young per litter was 6.9. At 2.5 mg., the litter size averaged 8.6. Larger amounts of  $\alpha$ -tocopherol did not influence size, which results are in agreement with those reported by Evans and Burr ('27) for wheat germ oil.

*Number of living young.* In litters of rats receiving 1.0 mg. of  $\alpha$ -tocopherol, 131 (67%) of 194 young were living at the time that the litters were found. As the amount of  $\alpha$ -tocopherol was increased up to 10 mg., the number of living young increased to 90%. Twenty and 50 mg. were no more effective than 10 mg.

Although no systematic study has been made of the newborn rats, dystrophic lesions of the skeletal muscles were discovered in nine of twenty-three rats dying or killed on the first day. Oedema, with or without necrosis of muscle fibers, was noted in a large proportion of these newborn rats.

*Number of second and third litters.* The birth of second litters after one dose of vitamin E has been reported by Evans and Burr ('27) and others. The number of second and third litters born after a single dose of  $\alpha$ -tocopherol at the beginning of the first gestation is shown in table 3. Since there was no apparent difference between them, the data obtained from the three separate preparations were combined in this and the

TABLE 3

*Number of litters occurring in rats on an E-low diet after a single dose of  $\alpha$ -tocopherol at the beginning of the first gestation.*

NUMBER OF LITTERS.	MILLIGRAMS OF $\alpha$ -TOCOPHEROL					
	0.5	1.0	2.5	10.0	20.0	50.0
1	0	12	13	10	14	6
2		3	4	5	6	1
3		1	0	2	1	1
4		0		0	0	0

following tables. It will be seen that as the  $\alpha$ -tocopherol was increased in amount from 1.0 to 10 mg., the proportion of second litters became greater, but at no level was it higher than 50%. Third litters occurred but rarely, even at 20 and 50 mg., and there were no fourth litters.

From the evidence it appears that the ability of female rats to produce more than one litter after a single dose of  $\alpha$ -tocopherol is limited and that the saturation point is reached at approximately 5 to 10 mg. These results are not entirely in accord with those of Evans and Burr ('27) who state that "the administration of the minimal effective dose of E never leads to the survival of fertility beyond the trial gestation for which it was employed", or of Olcott and Mattill ('34) who report that a single large dose of vitamin E allows fertility for two gestations but not for three.

*Success of lactation*

*Survival of young.* The number of young which survived the first 10 days of lactation is given in table 2. It will be seen that about half of the young of litters of females receiving 1.0 to 10 mg. of  $\alpha$ -tocopherol at the beginning of gestation survived. At 20 to 50 mg. about 70% survived. These results are in agreement with those of Sure ('26) who reported upon the existence of a factor in the unsaponifiable matter from wheat germ oil, necessary for successful lactation.

The cause of death of the young rats from the second to the tenth day was not determined; only in a few instances, were there any muscle lesions.

*Incidence of muscular dystrophy.* In table 2 is shown the incidence of muscular dystrophy in the young of females receiving single doses of  $\alpha$ -tocopherol at the beginning of gestation. At 1.0 mg., all of the young (sixteen) developed the disorder. With larger doses there was some protection, but even at 50 mg. the incidence of muscle disease was 76%. These results indicate that the capacity of female rats to store vitamin E given at the beginning of gestation, to transfer it through the placenta, and to excrete it in the milk, is so limited that their young are not protected against muscle dystrophy. The disease is prevented in all of the young if 10 mg. of  $\alpha$ -tocopherol are given to the female at the time of parturition (Evans and Emerson, '40).

*Prevention of muscular dystrophy in the young rat*

*Preparation of test animal.* The reproductive performance of female rats on diet I with 1 gm. of fresh wheat germ oil during the first 5 days of each gestation is given in table 4. The data are arranged according to the number of the gestations. Two hundred and thirty-seven gestations resulted in 214 litters, a fertility of 90%. The fertility of the rats during the first three gestations averaged more than 90%. In subsequent gestations, when the rat was 10 months of age or older, the fertility decreased, as reported by Olcott and Mattill ('34) and Emerson and Evans ('39).

The size of the litter tended to decrease in older animals, but the proportions of living young remained constant (averaging 90%). The numbers of living young were distributed among litters as follows: of 214 litters, there were 156 (73%) in which all of the young were living; 45 (22%) in which some of the young were living; and 12 (5%) in which none were living.

TABLE 4

*Reproduction of female rats on diet I with 1 gm. fresh wheat germ oil at the beginning of gestation.*

NUMBER OF GESTATION	1	2	3	4	5	6	TOTALS
No. of gestations	12	70	87	50	14	4	237
No. of litters	12	66	80	41	12	3	214
Per cent fertility	100	94	92	82	86	75	90
No. of young	86	537	565	244	68	10	1510
A.v. young per litter	7.2	8.1	7.1	6.0	5.7	3.3	7.0
No. living young	69	497	528	207	59	10	1370
Per cent	80	92	93	85	87	100	91
No. surviving 10 days	51	401	432	177	52	8	1121
Per cent	74	81	82	85	88	80	82
No. kept for muscular dystrophy study (not given $\alpha$ -tocopherol)	22	190	206	86	22	4	530
No. with disease	22	162	184	83	19	2	472
Per cent	100	85	88	96	86	50	89

The number of young surviving 10 days of lactation also remained constant (averaging 80%). They were distributed among litters as follows: of 202 litters of living young, there were 93 (46%) in which all of the young survived; 75 (37%) in which part of the young survived; and 34 (17%) in which none of the young survived. The number of young surviving 10 days of lactation was greater than had been observed upon 50 mg. of  $\alpha$ -tocopherol (see table 2).

However, in forty-four females on diet I that were fed 20 mg. of  $\alpha$ -tocopherol at the beginning of gestation (not the first gestation), forty-three (98%) had litters; there were 334 young (average 8.0 per litter), of which 311 (93%) were

living; 259 (83%) survived. These results compare favorably with those of the rats receiving wheat germ oil.

The incidence of muscular dystrophy at the end of lactation among the young rats is shown in table 4. Four hundred and seventy-two (89%) of 530 young developed the disorder. The rats with muscle lesions were distributed in litters as follows: of 165 litters in which four or more rats survived 10 days of lactation, there were 129 (78%) in which all of the rats presented degenerated muscles; 30 (18%) in which part of the young were affected and 6 (4%) in which none of the rats developed muscular dystrophy.

The occurrence of muscular dystrophy was determined by microscopic examination. In 863 rats with well-defined pathological lesions, 540 (63%) presented typical symptoms, and 805 (94%) presented gross lesions. Thus about a third of the animals had muscle lesions without symptoms. Calcification was observed in 72 (8%) of muscles. In 86 rats with slight microscopic lesions, 20 (23%) presented symptoms and only 27 (31%) presented gross pathological changes. Although the disease usually appeared on the nineteenth to twenty-third day of life, in one litter lesions were found in rats only 13 days of age.

*Prevention of muscular dystrophy in the young rat.* Muscular dystrophy is prevented in young rats of females on vitamin E-low diets by the feeding daily after the tenth day of lactation of small amounts of  $\alpha$ -tocopherol (Goettsch and Ritzmann, '39; Evans and Emerson, '40). In preliminary experiments single doses of approximately 0.5 mg. proved to be effective.

In order to determine at what time of lactation it was best to give the vitamin, single doses of 0.5 to 1.0 mg. of  $\alpha$ -tocopherol were fed to the young on the tenth to nineteenth day. The results are given in table 5. The greatest number of young were protected when the vitamin was fed on the fifteenth to seventeenth day. When fed at this time 0.5 mg. was as effective as 1.0 mg.

Varying amounts of the three preparations of  $\alpha$ -tocopherol were fed on the fifteenth to sixteenth day. It will be seen in table 6 that there were apparently no essential differences between the potencies of the natural and synthetic products. The minimum amount of  $\alpha$ -tocopherol which prevented muscular dystrophy in at least 85% of young under the given experimental conditions was approximately 0.5 mg.

TABLE 5

*Prevention of muscular dystrophy in the young rat by single doses of  $\alpha$ -tocopherol on the tenth to the nineteenth day of lactation*

DAY OF LACTATION	MILLIGRAMS $\alpha$ -TOCOPHEROL					
	1.0			0.5		
	No. of rats	No. with muscular dystrophy	%	No. of rats	No. with muscular dystrophy	%
10-11	42	7	17	36	11	30
12-14	12	1	8	21	3	14
15-17	49	1	2	54	1	2
18-19	19	0	0	11	2	18

TABLE 6

*Prevention of muscular dystrophy in the young rat by the feeding of single doses of  $\alpha$ -tocopherol on the fifteenth to sixteenth day of lactation.*

$\alpha$ -TOCOPHEROL	mg.	NUMBER OF RATS	NUMBER WITH MUSCULAR DYSTROPHY	PER CENT	
				PER CENT	PER CENT
Natural	0	278	255	92	92
Synthetic	0.25	9	3	33	33
Synthetic acetate		8	3	38	38
Natural	0.50	9	2	22	22
Synthetic		8	1	12	12
Synthetic acetate		85	8	9	9
Natural	1.00	18	0	0	0
Synthetic acetate		70	1	1.4	1.4
Synthetic acetate	2.00	6	0	0	0
Synthetic acetate	3.00	11	0	0	0

An assay method based upon the cure of muscle dystrophy in the young rat is not feasible because of the spontaneous recovery of about 65% of the affected young (Evans and Burr, '28; Goetsch and Ritzmann, '39).

*Relative ineffectiveness of parenteral administration.* Litter mates reared on diet I were divided into three groups. At the beginning of gestation one group was fed 1.0 mg. of  $\alpha$ -tocopherol (natural or the synthetic acetate), the second group was fed 2.5 mg., and the third group was given 2.5 mg. by subcutaneous injection. The results are shown in table 7. It will be seen that the rats receiving 2.5 mg. of  $\alpha$ -tocopherol subcutaneously responded in approximately the same way with respect to fertility, litter size, number of living young, and lactation as the rats that were fed 1.0 mg.

TABLE 7

*Ineffectiveness of parenteral administration of  $\alpha$ -tocopherol in the prevention of sterility in the female rat.*

	MILLIGRAMS OF $\alpha$ -TOCOPHEROL: SINGLE DOSE AT BEGINNING OF GESTATION		
	2.5	1.0	2.5
	Fed	Fed	Injected subcutaneously
No. of gestations	7	8	10
No. of litters	7	5	5
Per cent fertility	100	62	50
No. of young	67	36	39
Av. young per litter	9.6	7.2	7.8
No. living young	63	15	30
Per cent	94	42	77
No. surviving 10 days	42	12	10
Per cent	67	80	33
No. kept for muscular dystrophy study (not given $\alpha$ -tocopherol)	19	12	10
No. with disease	16	12	10
Per cent	84	100	100

Young rats of females on diet I were given  $\alpha$ -tocopherol (natural or synthetic acetate) orally or parenterally on the fifteenth to seventeenth day of lactation. By separating the young for several hours it was possible to prevent the mother rats from obtaining  $\alpha$ -tocopherol from the sites of injection. It will be seen in table 8 that although the young which were fed 0.5 or 1.0 mg. of  $\alpha$ -tocopherol were protected, their litter mates which received the same amounts by injection developed

muscle lesions. The parenteral administration of larger amounts of  $\alpha$ -tocopherol afforded some protection but the injection of 10 mg. subcutaneously or intraperitoneally was not so effective as the feeding of 0.5 mg.

TABLE 8

*Failure to prevent muscular dystrophy in the young rat by the injection of single doses of  $\alpha$ -tocopherol on the fifteenth to seventeenth day of lactation.*

	$\alpha$ -TOCOPHEROL	NUMBER OF RATS	NUMBER WITH MUSCULAR DYSTROPHY	PER CENT
Untreated controls	mg.			
	0	65	61	94
Subcutaneous injection	0.5	6	6	100
	1.0	17	15	88
	2.5	12	9	75
	5.0	9	6	67
	10.0	8	5	62
Intraperitoneal injection	0.5	2	2	100
	1.0	13	12	92
	2.5	6	6	100
	5.0	10	6	60
	10.0	8	3	38
Oral	0.5	17	1	6
	1.0	27	0	0

## SUMMARY

1. Under the experimental conditions described, 2.5 mg. of  $\alpha$ -tocopherol fed at the beginning of gestation permits the birth of young to at least 85% of female rats on vitamin E-low diets. The litters are of normal size (average 8.6 young), there are at least 85% of living young, 60% of which survive the first 10 days of lactation. Of the surviving rats, 90% develop muscular dystrophy at the end of lactation. Second litters are born to about one-fourth of the females.

2. Under the experimental conditions described, 0.5 mg. of  $\alpha$ -tocopherol fed on the fifteenth to the seventeenth day of lactation prevents muscular dystrophy in at least 85% of the young of female rats on vitamin E-low diets.

3. There are no essential differences in the anti-sterility and anti-muscular dystrophy preventing potencies between natural or synthetic  $\alpha$ -tocopherol or the synthetic acetate.
4. The  $\alpha$ -tocopherol must be fed as it is not so active when administered parenterally.
5. Muscular dystrophy may occur in the newborn rat.

#### LITERATURE CITED

- BACHARACH, A. L. 1938 Investigations into the method of estimating vitamin E. III. The relation between dosage and response to vitamin E. *Biochem. J.*, vol. 32, p. 2017.
- BACHARACH, A. L., AND E. ALLOCORNE 1938 Investigations into the method of estimating vitamin E. II. Further observations on vitamin E deficiency and implantation. *Biochem. J.*, vol. 32, p. 1298.
- EMERSON, G. A., AND H. M. EVANS 1939 Restoration of fertility in successively older E-low female rats. *J. Nutrition*, vol. 18, p. 501.
- EVANS, H. M. 1928 Spontaneous deciduomata in pseudopregnancy with low vitamin E. *Amer. J. Physiol.*, vol. 85, p. 149.
- EVANS, H. M., AND K. S. BISHOP 1923 On the relation between fertility and nutrition. IV. The production of sterility with nutritional regimes adequate for growth. *J. Metabolic Res.*, vol. 3, p. 233.
- EVANS, H. M., AND G. O. BURR 1927 The antisterility vitamine fat soluble E. *Memoirs of the University of California*, Berkeley, California, vol. 8.
- 1928 Development of paralysis in the suckling young of mothers deprived of vitamin E. *J. Biol. Chem.*, vol. 76, p. 273.
- EVANS, H. M., AND G. A. EMERSON 1940 Prevention of nutritional muscular dystrophy in suckling E-low rats with  $\alpha$ -tocopherol and related substances. *Proc. Soc. Exper. Biol. and Med.*, vol. 44, p. 636.
- EVANS, H. M., O. H. EMERSON AND G. A. EMERSON 1936 The isolation from wheat germ oil of an alcohol,  $\alpha$ -tocopherol, having the properties of vitamin E. *J. Biol. Chem.*, vol. 113, p. 319.
- GOETTSCH, M., AND J. RITZMANN 1939 The preventive effect of wheat germ oils and of  $\alpha$ -tocopherol in nutritional muscular dystrophy of young rats. *J. Nutrition*, vol. 17, p. 371.
- HAWK, P. B., AND B. L. OSER 1931 A modification of the Osborne-Mendel salt mixture. *Science*, vol. 74, p. 369.
- KARRER, P., AND H. KELLER 1938 Quantitative Bestimmung der Tocopherole in verschiedenen Ausgangsmaterialien. *Helv. chim. Acta*, vol. 21, p. 1161.
- MASON, K. E. 1940 Minimal requirements of male and female rats for vitamin E. *Am. J. Physiol.*, vol. 131, p. 268.
- MASON, K. E., AND W. L. BRYAN 1938 Standardization of the rat for bio-assay of vitamin E. *Biochem. J.*, vol. 32, p. 1785.
- MATTILL, H. A., AND N. C. STONE 1923 II. The nutritive properties of milk with special reference to reproduction in the albino rat. *J. Biol. Chem.*, vol. 55, p. 443.

- OLCOTT, H. S. 1938 The paralysis in the young of vitamin E deficient female rats. *J. Nutrition*, vol. 15, p. 231.
- OLCOTT, H. S., AND H. A. MATTILL 1934 Vitamin E. I. Some chemical and physiological properties. *J. Biol. Chem.*, vol. 104, p. 423.
- PALMER, L. S. 1937 Biological assay of vitamin E; application to wheat germ and wheat germ oil. *Indust. Eng. Chem. Anal. Ed.*, vol. 9, p. 427.
- SURE, B. 1923-1924 II. The existence of a specific vitamin for reproduction. *J. Biol. Chem.*, vol. 58, p. 693.
- 1926 Dietary requirements for reproduction. VII. The existence of a lactation-promoting factor in the unsaponifiable matter from wheat germ oil. *J. Biol. Chem.*, vol. 69, p. 53.

## THE UTILIZATION OF THE CALCIUM OF CAULIFLOWER AND BROCCOLI

MARGARET L. FINCKE

*School of Home Economics, Oregon State College, Corvallis*

(Received for publication July 24, 1941)

The availability of the calcium of several vegetables has been studied in various laboratories. Fincke and Sherman ('35) reviewed the literature on the subject up to 1935. They reported that the calcium of kale was practically as well utilized as that of milk, whereas the calcium of spinach was poorly utilized by young growing rats, if, indeed, it was utilized at all. The poor utilization of the calcium of spinach was due, in part at least, to the high oxalate content. Fairbanks and Mitchell ('38) confirmed these findings for spinach.

Kung, Yeh and Adolph ('38) studied several Chinese vegetables and found that with the exception of spinach and soybean sprouts, all caused high calcium retention values, which were comparable with those obtained with skim milk powder. Kao, Conner and Sherman ('38) reported that the availability of the calcium of Chinese cabbage was 90% as high as that of milk. Speirs ('39) found the calcium of turnip greens to be as available as that of milk and that of tendergreen, collards and kale somewhat less. The calcium of New Zealand spinach not only was poorly utilized by rats, if at all, but the utilization of the calcium of milk in the diet was diminished by the presence of the New Zealand spinach. The dried New Zealand spinach contained 4.8% oxalate. More recently this author ('40) has suggested that some factor other than oxalic acid may be concerned with this poor utilization.

The calcium of carrots, lettuce, and string beans was reported by Shields, Fairbanks, Berryman and Mitchell ('40) to be somewhat less available than that of milk. With milk solids, 77 to 83% of the calcium was retained, while 71% of the calcium of carrots, 61% of that of lettuce, and 60% of the calcium of string beans was utilized, with no difference being observed between fresh and cooked vegetables so far as calcium utilization was concerned.

This investigation was undertaken to study the utilization of calcium of two vegetables which are flowers, i.e., cauliflower and sprouting broccoli.

#### EXPERIMENTAL

The method here used is essentially that carried out by Fincke and Sherman ('35). Healthy young rats were weaned at 28 days and placed on the experimental diets, the compositions of which are given in table 1.

TABLE 1  
*Percentage composition of diets, and calcium content.*

	DIET 108	DIET 109	DIET 110
Whole wheat	44.5	44.5	44.5
Skim milk powder	20.0	10.0	10.0
Butterfat	10.0	10.0	10.0
Cornstarch	24.5	20.5	—
Dried broccoli	—	14.0	—
Dried cauliflower	—	—	34.5
Sodium chloride	1.0	1.0	1.0
Calcium content in %	0.277	0.267	0.267

Litter mates of the same sex were used in comparing the three diets, one animal from each litter being placed on each diet and one killed at 28 days of age for analysis. The initial weights of the animals were kept as nearly alike as possible. Each group consisted of five males and five females. The diets were fed ad libitum, records being kept of the amounts eaten until the animals were 60 days of age, when they were killed by chloroform. In previous unpublished work, no differences

were observed between the results obtained with ad libitum feeding and paired feeding. The bodies of the animals minus the digestive tract were ashed and analyzed for calcium by a modification of the McCrudden method.

Those parts of cauliflower and broccoli used for human consumption were prepared in amounts sufficient to last through the whole experiment as follows: washed well in tap and then distilled water, dried in a recirculating air drier operated at temperatures not exceeding 60°C., ground and thoroughly mixed. Each vegetable was carefully sampled and analyzed for calcium.

#### RESULTS

The comparative growth of the animals is given in table 2. Growth of the rats which were fed the diet containing broccoli amounted to 0.255 gm. per gram of food for the males, and 0.226 gm. per gram of food for the females. These gains were somewhat less than those of the rats fed the control diet (0.285 gm. and 0.248 gm. per gram of food for the males and

TABLE 2

*Growth and calcium content of animals receiving diets containing milk, or milk plus broccoli or cauliflower as chief sources of calcium.*

*All values are averages.*

DIET	SEX					CALOTUM			
		BODY WEIGHT		FOOD EATEN	GROWTH PER GRAM OF FOOD EATEN	Body content		Ingested	Utilization factor
		Initial	Final			gm.	Per cent		
108	♂ (5) <sup>1</sup>	49.4	150.4	355	0.285	1.275	0.848	0.982	0.87
	♀ (5)	49.6	131.6	331	0.248	1.224	0.932	0.917	0.87
$0.87 \pm 0.017^2$									
109	♂ (5)	49.8	137.4	344	0.255	1.151	0.839	0.918	0.79
	♀ (5)	49.8	123.8	328	0.226	1.111	0.898	0.877	0.79
$0.79 \pm 0.018^2$									
110	♂ (5)	49.8	111.6	288	0.212	0.989	0.892	0.769	0.73
	♀ (5)	50.4	99.6	272	0.180	0.916	0.920	0.728	0.66
$0.69 \pm 0.020^2$									

<sup>1</sup> Number of animals indicated in parentheses.

<sup>2</sup> Average and standard error of the mean.

females respectively), although food consumption was practically the same. The difference is not statistically significant, however. Food consumption and growth were significantly lower on the diet containing cauliflower than on the control diet, the growth per gram of food being 0.212 gm. for the males and 0.180 gm. for the females, or 26% less on the cauliflower diet. Probably some of this difference was due to the presence of fiber, as less efficient growth on diets containing fiber was observed by Fincke ('35). In those studies the gain per gram of food was depressed by 22% by the simple addition of 1.2% washed fiber to the diet consisting of two-thirds whole wheat, one-third whole milk powder.

The utilization of calcium of the different diets is shown in table 2. The calcium utilization factor here given is obtained by dividing the amount of calcium stored during the 32 days on the diet by the amount of calcium ingested. The storage of calcium represents the amount of calcium in the body of the rat at death minus the amount in the body at 28 days of age, calculated from the average calcium content of the 28-day old rats of the same sex which were killed for analysis at that age. The males at this age contained on the average 0.861% and the females 0.865% calcium.

Little difference is evident in the average percentages of calcium in the bodies of the rats on the three different diets. However, the animals receiving the broccoli or cauliflower diets were less efficient in their use of calcium, the differences being statistically significant. The milk calcium apparently was 87% available, that of the broccoli plus milk was 79% available, and that of the cauliflower plus milk only 69% available. In other words, the calcium utilization factor of the broccoli diet was 8.9% lower than that of the milk diet, and that of the cauliflower diet was 19.1% less than that of the milk diet. On comparing the percentage utilization of calcium of milk here obtained with the figures reported by Fincke and Sherman (0.81 for males and 0.75 for females), by Fairbanks and Mitchell (0.90), by Shields, Fairbanks, Berryman and Mitchell (0.668 to 0.83), and by Speirs (0.80 and 0.78), it will

be seen that the figures here found are in the same range as those reported by other investigators.

The dried broccoli contained 0.38% oxalic acid and the dried cauliflower 0.24%, determined by a modification of Bau's method ('19). If these amounts of oxalate rendered equivalent amounts of calcium unavailable, the calcium utilization factors of the diets containing broccoli and cauliflower calculated on the basis of available rather than total calcium would average 0.80 for each diet, which is still somewhat below the value here obtained for the milk diet.

The crude fiber content of dried broccoli and cauliflower was determined by the A.O.A.C. method ('40) and was found to be 9.7% for the broccoli and 9.6% for the cauliflower. The cauliflower diet, however, contained much more dried vegetable than the broccoli diet, so that the fiber content of that diet was higher. Adolph, Wang and Smith ('38) reported that loss of calcium in the feces when large quantities of fiber were fed amounted to less than 10%. Former work by the author, including some unpublished data, showed a barely significant lowering of the availability of the calcium due to the presence of added fiber, the lowering amounting to about 8%. If the calcium utilization factor here found for the milk diet, 0.87, were lowered by 8%, it would result in a factor of 0.80. The combination of the fiber and the oxalate contents of the broccoli and cauliflower diets could, therefore, account for the total lowering of the availability of the calcium of these two diets, and may actually have been the causes of poorer utilization of the calcium of broccoli and cauliflower.

#### SUMMARY AND CONCLUSIONS

Young rats in strict litter mate comparison were fed diets in which practically all of the calcium was derived from dried milk, or in which half of the milk was replaced by dried broccoli or cauliflower to provide approximately the same amounts of calcium. At 60 days of age they were killed and their bodies analyzed for calcium.

The calcium utilization factor for the milk diet amounted to  $0.87 \pm 0.017$ ; of the diet containing broccoli to  $0.79 \pm 0.018$ , and of the diet containing cauliflower to  $0.69 \pm 0.020$ . The possible causes of this lower availability of the calcium of cauliflower and broccoli are discussed.

#### LITERATURE CITED

- ADOLPH, W. H., C. H. WANG AND A. H. SMITH 1938 The effect of roughage on the calcium balance in rats. *J. Nutrition*, vol. 16, pp. 291-297.
- ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1940 *Methods of Analysis*, 5th edition.
- BAU, A. 1919 Determination of oxalic acid. *Wochschr. Brau.*, vol. 36, p. 293; vol. 37, p. 201.
- FAIRBANKS, B. W., AND H. H. MITCHELL 1938 Availability of calcium in spinach, in skim milk powder and in calcium oxalate. *J. Nutrition*, vol. 16, pp. 79-89.
- FINCKE, M. L. 1935 The availability of calcium from some typical foods. Dissertation, Columbia University, New York.
- FINCKE, M. L., AND H. C. SHERMAN 1935 The availability of calcium from some typical foods. *J. Biol. Chem.*, vol. 110, pp. 421-428.
- KAO, H. C., R. T. CONNER AND H. C. SHERMAN 1938 The availability of calcium from Chinese cabbage (*Brassica pekinensis*, Rupr.). *J. Biol. Chem.*, vol. 123, pp. 221-228.
- KUNG, L. C., H. L. YEH AND W. H. ADOLPH 1938 The availability of calcium in vegetable food materials. *Chinese J. Physiol.*, vol. 13, pp. 307-316. *Nutr. Abs. Rev.*, vol. 8, p. 697 (1939).
- SHELTON, J. B., B. W. FAIRBANKS, G. H. BERRYMAN AND H. H. MITCHELL 1940 The utilization of calcium in carrots, lettuce and string beans in comparison with the calcium in milk. *J. Nutrition*, vol. 20, pp. 263-278.
- SPEIRS, MARY 1939 The utilization of the calcium in various greens. *J. Nutrition*, vol. 17, pp. 557-564.
- 1940 The availability of calcium in various foods. In *Notes on Graduate Studies and Research in Home Economics and Home Economics Education, 1939-1940*, U. S. Department of Agriculture.

## THE EFFECT OF INCUBATION ON THE VITAMIN CONTENT OF EGGS

ESMOND E. SNELL AND ERNESTINE QUARLES

*Department of Chemistry, University of Texas, Austin*

(Received for publication July 18, 1941)

Almost no work has been reported on the changes which occur in the vitamin content of hen eggs during incubation and hatching. Suomalainen ('39 a) found that the vitamin A content of incubated eggs decreased by 45% during the second and third weeks of incubation, while the carotene remained unchanged. The same author ('39 b) followed the changes in ascorbic acid content of eggs during incubation. Ascorbic acid was absent in the unhatched egg, but increased greatly during the first 2 weeks of incubation. Since the chick requires vitamin A in its diet, but is able to synthesize its own supplies of vitamin C, these are the changes which might have been expected. At the same time, however, such studies afford a means for determining which of the other vitamins can be synthesized by the developing chick embryo and presumably, therefore, are not required in the diet of the chick.

In the present study the total concentrations of riboflavin, pantothenic acid, nicotinic acid, biotin and inositol in the egg have been followed from the beginning of incubation to the time of hatching. Evidence is abundant and conclusive that riboflavin and pantothenic acid are required in the diet of chicks, and for development of the chick embryo (Lepkovsky et al., '38; Bauernfeind and Norris, '39; Jukes, '37), while biotin is presumably also required (Ringrose et al., '31; Hegsted et al., '40). No effect of nicotinic acid on chick growth has been observed (Mickelsen et al., '38). Inositol, recently identi-

fied as a dietary essential for the mouse (Woolley, '40), has not been studied in this regard. Pantothenic acid and riboflavin have therefore been used to indicate the changes which may occur in the concentration of an essential dietary constituent during embryonic development, and changes in the other constituents studied have been compared with these.

#### EXPERIMENTAL

The eggs used were from a private flock of White Leghorn breeders maintained near Austin, and showed uniformly high hatchability. The weighed eggs were marked and incubated at 38.9–39.5°C. in electrical units. At 3- to 4-day intervals throughout the incubation period eight eggs which showed normal embryonic development were taken. These were divided into four sets of two eggs each. The contents of the two eggs of each set were removed from the shells, combined, an equal weight of water added, and the sample homogenized in the Waring Blender. All samples were then allowed to autolyze under benzene at 37°C. for 24 hours. Both pantothenic acid (Rohrmann et al., '34) and biotin (Snell et al., '40) occur in tissues in a bound form liberated by autolysis. While this procedure was superfluous at the beginning of incubation it becomes increasingly necessary as the embryo develops, and was carried out in all cases to insure reproducibility of results. At the end of this time all samples were diluted to contain the equivalent of 200 mg. of original sample (including shell) per cubic centimeter. Since eggs lose considerable weight during incubation (compare Pringle and Barott, '37) all weights were expressed as the original egg weight before incubation. Aliquots were autoclaved at 15 pounds steam pressure for 20 minutes, then rediluted with water to the original volume. Glacial acetic acid was added dropwise with shaking until the coagulated material became flocculent and easily filtered. A portion of the filtrate was saved for analysis.

At the end of 20 days incubation some chicks were beginning to hatch. Unhatched chicks were taken for the 20-day

group. The hatched chicks taken at 23 days were from eggs of known weight and had not yet received food or water. Samples were prepared from the whole chicks as described above, except that the chick was decapitated and ground before being placed in the Blender. The average weight of the chicks of this group was 34.3 gm.; the average original weight of the eggs (before incubation) from which they were hatched was 57.5 gm.

Riboflavin was determined by the microbiological method (Snell and Strong, '39). Pantothenic acid was determined by the method of Snell, Strong and Peterson ('37) as modified by Pennington et al. ('40). Several samples were also assayed by the modification of the former method presented by Strong, Feeney and Earle ('40) with identical results. Nicotinic acid was determined microbiologically with *Lactobacillus arabinosus* (Snell and Wright, '41). Biotin was determined with this same organism, as recommended in the latter reference. A sample of biotin methyl ester (kindly furnished by Doctor Du Vigneaud) was hydrolyzed with alkali and used as the biotin standard. Inositol was determined by a yeast growth method (Williams et al., '41).

Results are given in table 1. Pantothenic acid remained constant throughout the incubation period. Riboflavin likewise remained constant. The small apparent increase in riboflavin during the first 4 to 7 days of incubation represented an extraction difficulty during the first few days, since direct assays made by adding the mixed original sample (without heating or filtering) to the assay tube gave figures in good agreement with those obtained from 7 days on. Thus two vitamins of known essential nature for the chick remain constant during incubation, neither increasing nor decreasing in total concentration. In marked and unmistakable contrast, nicotinic acid rises steadily from a low initial value to a level more than twenty times higher in the hatched chick. This demonstrates conclusively that the chick embryo synthesizes its own nicotinic acid, and that bacterial synthesis is not involved. Presumably this synthetic power is retained by the

TABLE 1  
*Effect of incubation on the vitamin content of eggs.*

INCUBATION TIME	EGG WEIGHT PER PAIR	PANTOTHENIC ACID	RIBO-FLAVIN	NICOTINIC ACID	INOSITOL	BIOTIN
days	gm.	μg. per gm.	μg. per gm.	μg. per gm.	μg. per gm.	μg. per gm.
0	120	12	2.0	0.77	74	59
	117	11	1.9	0.73	64	53
	117	10	2.3	0.90	73	70
	116	12	1.9	1.1	82	90
Average	118	11	2.0	0.85	73	68
4	113	12	3.2	1.1	57	50
	113	12	2.4	0.90	66	51
	117	13	2.5	0.90	69	50
	117	10	2.3	1.1	67	57
Average	115	12	2.6	1.0	65	52
7	117	14	3.0	2.7	66	92
	116	14	3.6	2.8	70	67
	113	13	3.4	3.0	78	69
	115	12	3.3	2.8	85	82
Average	115	13	3.3	2.8	75	78
10	119	14	3.5	3.1	88	99
	115	12	3.1	2.6	95	53
	117	13	3.0	2.6	89	86
	115	10	2.9	3.4	88	75
Average	116	12	3.1	2.9	90	78
13	119	14	2.9	6.9	104	62
	116	12	3.0	7.4	119	53
	115	12	2.8	8.0	121	54
	117	13	3.4	7.1	128	44
Average	117	13	3.0	7.3	118	53
17	118	11	3.0	13	200	46
	115	11	3.2	15	224	57
	116	13	3.4	17	208	76
	118	10	2.7	11	167	26
Average	117	11	3.1	14	200	51
20	116	11	3.2	17	269	64
	118	14	3.0	16	264	36
	119	16	3.8	15	266	68
	119	12	2.9	15	254	60
Average	118	13	3.2	16	263	57
23	111	10	3.3	16	445	26
	116	13	3.3	20	468	23
	116	11	2.9	19	490	29
	116	14	3.5	20	420	22
Average	115	12	3.3	19	456	25

chick. All efforts to raise the apparent nicotinic acid content of unincubated egg, or to alter the final value by acid or alkaline hydrolysis of the samples met with failure. Kringstad and Thoresen ('40) found by chemical assay only traces of nicotinic acid in hen eggs, although the flesh of hens was twice as rich in nicotinic acid as was beef. Dann and Handler ('41) have recently reported marked increases in the nicotinic acid content of chick breast muscle during the first 3 months after hatching. Shourie and Swaminathan ('40) have shown by balance experiments that rats do not require nicotinic acid in the diet. The possibility that it was furnished by bacterial action in the intestinal tract, and not synthesized by the tissues could not be excluded by this method.

In a manner similar to nicotinic acid, inositol increased regularly in concentration from about the seventh day of incubation on, to a final level about six times that present in the original egg. These results would indicate that the chick embryo is likewise capable of synthesizing inositol.

The biotin content of eggs was more variable than was any other constituent which was determined. The average biotin content, however, showed no tendency to increase during incubation, and decreased after hatching. The decrease is only apparent, however, since acid hydrolysis of the samples after autolysis liberated additional biotin to an average value of 42 my per gram. Acid or alkaline hydrolysis of the control egg samples did not increase their biotin content. From these results it is evident that the chick embryo does not synthesize biotin. This result might indicate merely that sufficient biotin was already present in the egg for development. The observation that the biotin level in chick tissues is lowered when biotin of the diet is rendered unavailable by egg white (Eakin, McKinley and Williams, '40) indicates that it is a true vitamin for the chick, and could not be synthesized under any conditions. Recent direct evidence (Hegsted et al., '40) also supports this conclusion.

## SUMMARY AND CONCLUSIONS

The total amount of pantothenic acid and of riboflavin present in hen eggs does not change during incubation. The amount of biotin also remains constant or decreases somewhat. Nicotinic acid is synthesized by the chick embryo, so that the final amount present in the newly-hatched chick is over twenty times that originally present in the unincubated egg. Inositol likewise appears to be synthesized by the chick embryo. Presumably these synthetic powers of the embryo are retained by the chick. If this is so, it would indicate that nicotinic acid and inositol, unlike riboflavin, pantothenic acid and biotin, are not required in the diet of the chick. Whether such synthetic powers are sufficient to supply the needs of the chick for optimal or maximal growth is, of course, not determined by the present data.

Generous support is acknowledged from funds granted to Prof. Roger J. Williams by the Rockefeller Foundation. The authors wish also to thank Mr. Raymond McMahan, Dr. L. D. Wright, and Dr. Alfred Taylor for assistance in certain parts of the work.

## LITERATURE CITED

- BAUERNFEIND, J. C., AND L. C. NORRIS 1939 The role of the antidermatosis vitamin and a new water soluble growth factor in the nutrition of the mature fowl. *J. Nutrition*, vol. 18, p. 579.
- DANN, W. J., AND P. HANDLER 1941 The quantitative estimation of nicotinic acid in animal tissues. *J. Biol. Chem.*, vol. 140, p. 201.
- EAKIN, R. E., W. A. MCKINLEY AND R. J. WILLIAMS 1940 Egg-white injury in chicks and its relationship to a deficiency of vitamin H (biotin). *Science*, vol. 92, p. 224.
- HEGSTED, D. M., J. J. OLESON, R. R. MILLS AND C. A. ELVEHJEM 1940 Studies on a dermatitis in chicks distinct from pantothenic acid deficiency. *J. Nutrition*, vol. 20, p. 599.
- JKES, T. H. 1937 Further observations on the assay, distribution, and properties of the filtrate factor. *J. Biol. Chem.*, vol. 117, p. 11.
- KRINGSTAD, H., AND F. THORESEN 1940 The occurrence of the antipellagra vitamin, nicotinic acid (-amide), in some foods. *Nord. Med.*, vol. 8, p. 2248.

- LEPKOVSKY, S., L. W. TAYLOR, T. H. JUKES AND H. J. ALMQUIST 1938 Effect of riboflavin and the filtrate factor on egg production and hatchability. *Hilgardia*, vol. 11, p. 559.
- MICKELSEN, O., H. A. WAISMAN AND C. A. ELVEHJEM 1938 The inactivity of nicotinic acid in chick dermatitis. *J. Biol. Chem.*, vol. 124, p. 313.
- PENNINGTON, D. E., E. E. SNELL AND R. J. WILLIAMS 1940 An assay method for pantothenic acid. *J. Biol. Chem.*, vol. 135, p. 213.
- PRINGLE, E. M., AND H. G. BAROTT 1937 Loss of weight of hen eggs during incubation under different conditions of humidity and temperature. *Poultry Sci.*, vol. 16, p. 49.
- RINGROSE, A. T., L. C. NORRIS AND G. F. HEUSER 1931 The occurrence of a pellagra-like syndrome in chicks. *Poultry Sci.*, vol. 10, p. 166.
- ROHREMAN, E., G. E. BURGET AND R. J. WILLIAMS 1934 Pantothenic acid content of animal tissues. *Proc. Soc. Exp. Biol. Med.*, vol. 32, p. 433.
- SHOURIE, K. L., AND M. SWAMINATHAN 1940 The synthesis of nicotinic acid by rats. *Indian J. Med. Research*, vol. 27, p. 679.
- SNELL, E. E., R. E. EAKIN AND R. J. WILLIAMS 1940 A quantitative test for biotin and observations regarding its occurrence and properties. *J. Am. Chem. Soc.*, vol. 62, p. 175.
- SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. *Ind. Eng. Chem. Anal. Ed.*, vol. 11, p. 346.
- SNELL, E. E., F. M. STRONG AND W. H. PETERSON 1937 Growth factors for bacteria. VI. Fractionation and properties of an accessory factor for lactic acid bacteria. *Biochem. J.*, vol. 31, p. 1789.
- SNELL, E. E., AND L. D. WRIGHT 1941 A microbiological method for the determination of nicotinic acid. *J. Biol. Chem.*, vol. 139, p. 675.
- STRONG, F. M., R. E. FEENEY AND A. C. EARLE 1940 Microbiological determination of pantothenic acid. *Abstracts, 100th Meeting Am. Chem. Soc.*, p. 21.
- SUOMALAINEN, P. 1939 a Vitamin A and carotene in the hen egg during hatching. *Suomen Kemistelehti*, vol. 12B, p. 30. *Chem. Abst.*, vol. 34, p. 145.
- 1939 b Synthesis of ascorbic acid (vitamin C) in the hen egg during hatching. *Ann. Acad. Sci. Fenniae*, vol. A53, no. 8. *Chem. Abst.*, vol. 34, p. 1724.
- WILLIAMS, R. J., J. R. MCMAHAN, A. K. STOUT AND H. K. MITCHELL 1941 An assay method for inositol. *Univ. of Texas Pub.* (In press.)
- WOOLLEY, D. W. 1940 The nature of the anti-alopecia factor. *Science*, vol. 92, p. 384.



## DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION

XXVIII. THE LACTATION-PROMOTING PROPERTIES OF CYSTINE WHEN  
ADDED TO CASEIN DIETS<sup>1</sup>

BARNETT SURE

*Department of Agricultural Chemistry, University of Arkansas, Fayetteville*

(Received for publication June 14, 1941)

As early as 1919 results of experiments conducted at the University of Wisconsin and published in 1924 (Sure, '24 a, b) disclosed that diets entirely satisfactory for growth and reproduction may be entirely inadequate for lactation. Later it became apparent (Sure, '27) that the requirements of the vitamin B complex for lactation are much greater than those for growth. That this is true for various components of the vitamin B complex became evident from quantitative studies using pure crystalline thiamine, riboflavin, pyridoxine, and choline (Sure, '40).

For a number of years various nutritional investigators have reported failures with rats, started on experiments from weaning, to rear their young when subsisting on purified diets devoid of natural foods. The extensive literature has been reviewed recently by Folley and associates ('38, '40) and therefore will be referred to only as it may be pertinent to the results of the studies submitted in this and the accompanying paper (no. XXIX of this series).

The preliminary report of Outhouse ('37), that a purified diet containing yeast, cod liver oil and wheat germ oil proved

<sup>1</sup> Research paper no. 699, Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

a complete failure for rearing of young whereas such a diet supplemented with cottonseed oil, oats, and lettuce resulted in successful lactation, stimulated the investigation of various fats and oils as possible lactogogues. Consequently, lard, butterfat, hydrogenated cottonseed oil,<sup>2</sup> olive oil, and hydrogenated cottonseed oil in combination with wheat germ oil,

TABLE 1  
*Percentage composition of rations.*

COMPONENT	RATION									
	16	16-a	17	17-a	18	18-a	19	19-a	20	20-a
Casein (purified) <sup>1</sup>	20	20	20	20	20	20	20	20	20	20
Salts no. 185 <sup>2</sup>	4		4		4		4		4	
Salts no. 351 <sup>3</sup>		4		4		4		4		4
Dried yeast <sup>4</sup>	10	10	10	10	10	10	10	10	10	10
Lard	15	15								
Butterfat			15	15						
Hydrogenated cottonseed oil <sup>5</sup>					15	15			10	10
Olive oil							15	15		
Wheat germ oil									5	5
Starch	51	51	51	51	51	51	51	51	51	51

<sup>1</sup> Commercial casein was obtained from Atterbury Brothers, New York City. This was purified by washing for 10 days with acidulated water and then by several washings with 25% ethanol at room temperature; then dried at 85 to 90°C. By analysis this product contained 13.85% N, or 88.4% protein when Jones' ('31) factor of 6.38 is used. Therefore the rations actually contained 17.7% protein from this casein instead of the 20% intended. Analysis revealed that the yeast contributed 3.7% additional protein making the total come to 21.4%.

<sup>2</sup> McCollum and Simmonds ('18).

<sup>3</sup> Hubbell, Mendel and Wakeman ('37).

<sup>4</sup> Northwestern Yeast Co., Chicago, Ill.

<sup>5</sup> Crisco.

were incorporated to the extent of 15% in the diet, and studies were made of their influence on fertility and lactation. Realizing that a mineral mixture entirely adequate for optimum success for reproduction and lactation had not as yet been found, this study was carried out with two salt mixtures, that of McCollum and Simmonds, no. 185 ('18) and that of Hubbell,

<sup>2</sup> Crisco.

Mendel and Wakeman, no. 351 ('37). In each experiment there were two males and four females, making a total of twenty males and forty females used. The animals were started after weaning, with initial weights ranging from 60 to 80 gm. The

TABLE 2  
*Lactation-promoting properties of cystine.*

RATION NUMBER	NUMBER OF MOTHERS	TOTAL YOUNG BORN	NUMBER OF STILL-BIRTHS	YOUNG GIVEN TO REAR	YOUNG REARED	LACTATION EFFICIENCY INDEX
<b>16</b>						
With cystine	4	40	0	24	24	100
Without cystine	2	19	10	6	0	0
<b>16-a</b>						
With cystine	4	47	0	30	29	97
<b>17</b>						
Without cystine	4	37	0	24	4	17
<b>17-a</b>						
With cystine	3	47	0	30	30	100
Without cystine	1	11	11			
<b>18</b>						
With cystine	2	16	0	11	11	100
Without cystine	2	18	0	12	0	0
<b>18-a</b>						
With cystine	3	39	0	24	22	91
Without cystine	2	43	14	22	0	0
<b>19</b>						
Without cystine	4	89	17	46	10	22
<b>19-a</b>						
With cystine	4	64	0	42	41	98
Without cystine	1	10	0	6	0	0
<b>20</b>						
With cystine	2	47	0	24	22	91
Without cystine	3	47	0	30	11	37
<b>20-a</b>						
With cystine	3	52	0	30	30	100
Without cystine	2	28	0	18	0	0

rations were planned to contain 20% casein,<sup>3</sup> but actually had 21.4% (see footnotes to table 1). Table 1 gives the percentage composition of the diets and the results on fertility and lactation are presented in table 2.

<sup>3</sup> Supplied by Atterbury Brothers, New York City.

Six drops of cod liver oil<sup>4</sup> as a source of vitamins A and D were given daily to each animal during growth and pregnancy, and 10 drops daily to each mother during lactation. Six to 8 drops of wheat germ oil as a source of vitamin E were given daily to each animal during growth and pregnancy, and 10 drops wheat germ oil daily to each mother during lactation.

Early in this investigation it became apparent that, regardless of the character of fats or oils or composition of salts employed, the mothers were failing to nurse their litters satisfactorily during the first 7 to 10 days and frequently during the first 3 days of lactation. At that time the work of Daggs and Lidfeldt ('38) appeared on the effect of sulphydryl compounds on milk production. They reported that the sulphydryl amino acids existing either as cysteine or in the potential form, cystine or methionine, are dietary lactogogues. The lactogenic potency of oxidized or reduced glutathione could be correlated with the respective contents of cystine or cysteine. Daggs ('35) and Daggs and Toumboulian ('35) had previously demonstrated that the lactation-augmenting effect of high protein diets, liver extracts, etc., is due to the extra amount of cystine or glutathione contained in the diet.

Based on Daggs' findings, an intensive effort was made to prevent failure in lactation by supplementation with additional amounts of cystine. The cystine was incorporated in the ration to the extent of 0.2%. This introduced 30 to 40 mg. daily additional cystine based on 15 to 20 gm. daily food intake during lactation. In some experiments the yeast was removed from the ration and 40 mg. cystine incorporated in a 1500 mg. daily yeast dose, as a source of the vitamin B complex. In two cases 20 mg. daily doses were given. Either the cystine administrations in the yeast or the diets containing cystine were given to the mothers when the growth curves of the nursing young reached a plateau or when abnormal gains were being made early in lactation. Success followed every one of thirty-seven trials. Out of 352 young born to mothers that received cystine as a supplement to the 17.7% casein diets,

<sup>4</sup> Mead-Johnson.

there were no stillbirths, and 209 out of 215 allowed to nurse were weaned, which means a lactation efficiency index of 97%. We consider 21 to 24 days to be a normal lactation period on our best stock diets, and only three mothers showed a prolonged lactation period of 27 to 29 days. On the other hand, out of 282 young born to mothers subsisting on the casein diets without provision of supplementary cystine, there were fifty-two stillbirths and only twenty-five out of 164 young allowed to nurse were weaned, or a lactation efficiency index of only 15%. In several cases lactating mothers were entirely successful with first litters when they received supplementary cystine and entirely failed with second litters when they were denied additional cystine administrations.

When the 1938 paper of Daggs and Lidfeldt appeared and it was then decided to add cystine to the diets, a number of females had already failed with their first litters while on diets not fortified with cystine; and, since the purpose of this investigation was to test the lactation stimulating effect of various fats and oils on two different salt mixtures, no systematic distribution of animals on each diet with respect to cystine additions and omissions was possible. It was clear, however, that regardless of the character of fats or oils or the composition of the salt mixture used, cystine was the limiting factor in all of the rations tested.

#### DISCUSSION

The role of cystine in nutrition was first investigated by Osborne and Mendel ('15). They found that when casein is incorporated in an otherwise adequate diet to the extent of 18%, young rats grow at a rate considered normal at that time. When, however, the proportion of casein was less than 15%, growth was subnormal, and at a level of 9% growth appeared at approximately half the normal rate. The addition of cystine to the ration was followed by accelerated growth of the experimental animals.

Recently Pavcek and Baum ('41) reported that better growth is observed when an 18% casein diet is supplemented

with 75 mg. cystine daily. It is not surprising, therefore, that cystine supplemented our 17.7% casein diet for lactation. Moreover, the beneficial results of cystine in lactation reported in this paper are in agreement with those of Wright and Haag ('39) who found that the lactation-promoting properties of rations containing approximately 9% of alfalfa crude protein are markedly enhanced by 0.4% addition of l-cystine.

In view of the fact that methionine can be converted to cystine (Beach and White, '37; Tarver and Schmidt, '39) and that the presence of cystine does not improve the quality of a ration containing a sufficiency of methionine (Womack, Kemmerer and Rose, '37), similar results in lactation may be anticipated from methionine additions to diets containing about 18% casein as the only source of protein.

The results of this study do not necessarily show the essential nature of cystine but do show that the lactation-promoting properties of a diet containing about 18% casein are markedly improved by the addition of this amino acid. Since casein contains 3.0 to 3.3% methionine, the rations provided about 0.6% of this amino acid, but apparently this amount is insufficient for rearing of young and needs supplementary cystine for successful lactation.

#### SUMMARY

The purpose of this investigation was to determine the lactation-promoting properties of various fats and oils, such as lard, butterfat, hydrogenated cottonseed oil, olive oil, and wheat germ oil in combination with hydrogenated cottonseed oil, fed at a 15% level on two types of salt mixtures. The rations contained 17.7% purified casein and 3.7% protein derived from the dehydrated bakers' yeast used as a source of the vitamin B complex. The albino rats used in this study were started on the experiments soon after weaning at initial weights ranging from 60 to 80 gm.

Regardless of the nature of the fats or oils, or the composition of the salt mixture, such diets did not meet the demands of lactation. The limiting factor in the rations was

found to be cystine. When the rations were fortified with 0.2% cystine, or daily administration of 20 to 40 mg. cystine to the lactating mothers was begun as failure of lactation became evident, lactation proceeded successfully and the young were weaned.

The results of this study do not necessarily prove the essential nature of cystine, but do show that the lactation-promoting properties of a diet containing about 18% casein are markedly improved by the addition of this amino acid. Since casein contains 3.0 to 3.3% methionine, the rations provided about 0.6% of this amino acid, but apparently this amount is insufficient for rearing of young and supplementary cystine can meet the need for successful lactation.

#### LITERATURE CITED

- BEACH, E. F., AND A. WHITE 1937 Synthesis of cystine by the albino rat. *J. Biol. Chem.*, vol. 127, pp. 87-95.
- DAGGS, R. G. 1935 Technic for studying lactation in rats. *J. Nutrition*, vol. 9, pp. 575-580.
- DAGGS, R. G., AND R. L. TOUMBOULIAN 1935 Effect of various dietary principles on lactation in rats. *J. Nutrition*, vol. 9, pp. 581-593.
- DAGGS, R. G., AND V. S. M. LIDFELDT 1938 The effect of the sulphhydryl compounds on milk production. *J. Nutrition*, vol. 15, pp. 211-221.
- FOLLEY, S. J., E. W. IKIN, S. K. KON AND H. M. S. WATSON 1938 Observations on specific nutritional factors in lactation. *Biochem. J.*, vol. 32, pp. 1988-2000.
- 1940 Lactation. *Biol. Rev.*, vol. 15, p. 421.
- HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture in experimental diets. *J. Nutrition*, vol. 14, pp. 273-286.
- JONES, D. B. 1931 Factors for converting percentage of nitrogen in foods and feeds into percentages of proteins. U. S. D. A. Circular No. 183, pp. 1-22.
- MCCOLLUM, E. V., AND N. SIMMONDS 1918 A study of the dietary essential, water-soluble B, in relation to solubility and stability towards reagents. *J. Biol. Chem.*, vol. 38, pp. 55-89.
- OSBORNE, T. B., AND L. B. MENDEL 1915 The comparative nutritive value of certain proteins in growth and the problem of the protein minimum. *J. Biol. Chem.*, vol. 20, pp. 351-378.
- OUTHOUSE, J. 1937 Rep. Ill. Agr. Exp. Sta., 1935-36, p. 285.
- PAVCEK, P. L., AND H. M. BAUM 1941 Relation of cystine to achromotrichia. *Proc. Soc. Exp. Biol. and Med.*, vol. 47, pp. 271-272.

- SURE, B. 1924 a The existence of a specific vitamin for reproduction. *J. Biol. Chem.*, vol. 58, pp. 693-710.
- 1924 b The existence of the reproductive dietary complex (vitamin E) in the ethereal extracts of yellow corn, wheat embryo, and hemp seed. *J. Biol. Chem.*, vol. 62, pp. 371-397.
- 1927 Vitamin B requirements for normal lactation. *J. Biol. Chem.*, vol. 74, pp. 55-70.
- 1940 Quantitative requirements of the components of the vitamin B complex for lactation and growth of nursing young of the albino rat. *J. Nutrition*, vol. 19, pp. 57-70.
- TARVER, H., AND C. L. A. SCHMIDT 1939 The conversion of methionine to cystine. *J. Biol. Chem.*, vol. 130, pp. 67-81.
- WOMACK, M., K. S. KEMMERER AND W. C. ROSE 1937 The relation of cystine and methionine to growth. *J. Biol. Chem.*, vol. 121, pp. 403-411.
- WRIGHT, L. D., AND J. R. HAAG 1939 The lactation-promoting effect of l-cystine when fed with alfalfa proteins. *J. Nutrition*, vol. 17, pp. 263-268.

## DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION

### XXIX. THE EXISTENCE OF A NEW DIETARY FACTOR ESSENTIAL FOR LACTATION<sup>1</sup>

BARNETT SURE

*Department of Agricultural Chemistry, University of Arkansas, Fayetteville*

(Received for publication June 14, 1941)

Before the existence of a new dietary factor essential for lactation could be established the quantitative requirements of the various known components of the vitamin B complex in pure form had to be determined. The results of such a study were recently published (Sure, '40). At that time pure pantothenic acid was not available, and the stimulating effect of the "W" factor extracts undoubtedly was due in part to pantothenic acid (Black, Frost and Elvehjem, '40).

It was realized that with the procedure involving transfer of experimental animals from stock to purified diets, during the short period of depletion of the reserves of the vitamin B complex there may be storage of one or more of these dietary essentials. Therefore, an extensive study was made of the fertility and lactation requirements for the various components of the B complex starting with albino rats at weaning, 25 to 28 days of age, and weighing 60 to 80 gm. each. Two males and four females were taken for each experiment. The basal diets and the supplements used are given in table 1.

<sup>1</sup> Research paper no. 721, Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station. Aided by a grant from the Committee on Scientific Research of the American Medical Association.

The females were bred at 80 to 90 days of age when they weighed 160 gm. or over, at which time the daily doses of supplements were increased as indicated in table 1. On the day of birth of the young these daily doses of supplements were again increased and these dosages maintained throughout lactation (table 1). Early in this study the rations contained 15% of hydrogenated cottonseed oil, but with the exception of ration C a change was made later to 10% butterfat,

TABLE 1

*Percentage composition of basal rations, and amounts of supplements used.*

COMPONENT	BASAL RATION					
	A	B	C	D	E	F
Casein (purified) <sup>1</sup>	20.00		20.00		20.00	10.00
Blood fibrin <sup>2</sup>		20.00		20.00		10.00
Dextrin	60.65	60.95	55.65	60.95	60.75	60.75
Butterfat	10.00	10.00		10.00	10.00	10.00
Crisco			15.00			
Hubbell-Mendel-Wakeman salts no. 351 <sup>3</sup>	4.00	4.00	4.00			
Sure's salts no. 1 <sup>4</sup>				4.00	4.00	4.00
Wheat germ oil	3.00	3.00	3.00	3.00	3.00	3.00
Agar-agar	2.00	2.00	2.00	2.00	2.00	2.00
Cystine	0.30		0.30		0.20	0.20
Nicotinic acid	0.05	0.05	0.05	0.05	0.05	0.05
SUPPLEMENTS	FROM WEANING TO MATING		DURING PREGNANCY		DURING LACTATION	
Thiamine <sup>5</sup>	20 µg.		50 µg.		120 µg.	
Riboflavin <sup>5</sup>	20 µg.		50 µg.		120 µg.	
Pyridoxine <sup>5</sup>	20 µg.		50 µg.		120 µg.	
Choline chloride <sup>5</sup>	6 mg.		9 mg.		15 mg.	
"W" extract	0.2 gm. <sup>6</sup>		0.5 gm. <sup>6</sup>		1.0 gm. <sup>6</sup>	
Vitamin K <sup>7</sup>	100 µg.		250 µg.		600 µg.	
Halibut liver oil	2 drops <sup>8</sup>		2 drops <sup>9</sup>		2 drops <sup>9</sup>	

<sup>1</sup> Thoroughly extracted with acidulated water and dilute ethanol.

<sup>2</sup> Thoroughly extracted with 95% ethanol.

<sup>3</sup> Hubbell, Mendel and Wakeman ('37).

<sup>4</sup> Composition given in table 4.

<sup>5</sup> Daily.

<sup>6</sup> Equivalent to this weight of original liver extract.

<sup>7</sup> Given as 2-methyl, 1-4 naphthoquinone.

<sup>8</sup> Once weekly.

<sup>9</sup> Three times weekly.

because of the report by Schantz, Elvehjem and Hart ('40) that butterfat is superior to vegetable oils homogenized into skinned milk in meeting the nutritive demands of early growth. All rations except B and D, which contained 20% blood fibrin, were fortified with 0.2 to 0.3% cystine from the time of mating.

The experiments may be divided into two series, I in which the Hubbell-Mendel-Wakeman salt mixture no. 351 ('37) was used, and II in which a modification of the Phillips and Hart ('35) salt mixture was employed (referred to as Sure's salts no. 1). The findings of these studies are summarized in tables 2, 3 and 5. Table 4 gives the new salt mixture.

### *Series I experiments*

These experiments were conducted on rations A, B and C which contained the Hubbell-Mendel-Wakeman salt mixture. Ration B used in this series of experiments and ration D used in series II experiments contained blood fibrin<sup>2</sup> (table 1). Beef-blood fibrin was used because of the report by J. H. Jones ('38) that this protein can support good growth at comparatively low levels of supply and also because it contains 3.5% cystine as well as 2.5% methionine (Toennies, '37) and therefore should not require any supplementation with cystine. After thorough extraction with hot 95% ethanol our fibrin preparation had a nitrogen content of 13.5%. Using the factor 5.92 (D. B. Jones, '31) our intended 20% level of supply of protein in the form of this fibrin in rations B and D proved to be 16%.

Experiments were initiated with and without addition of pure crystalline calcium pantothenate (table 2). It will be noted that reproduction was very abnormal, 46% of the young having been born dead; it also appears that supplementation with as much as 250 µg. daily of calcium panto-

<sup>2</sup> Since the work reported in this paper has been completed, Hegsted and associates ('41) have published a modification of the Phillips and Hart salt mixture, increasing the manganese sulfate from 0.7 gm. to 10 gm. and changing the K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O to the anhydrous salt.

TABLE 2

*Series I experiments. Control experiments with and without calcium pantothenate (rations A and B), and responses in lactation to administration of rice bran and liver extracts.*

NUMBER OF FEMALE MATED	RATION	DAILY DOSE OF CALCIUM PANTOTHENATE		TOTAL YOUNG BORN	NUMBER OF STILLBIRTHS	NUMBER OF STERILITY	YOUNG GIVEN MOTHERS TO NURSE	YOUNG REARED	REMARKS
		During pregnancy	During lactation						
9	A	0	0	38	19	4	15	0	One mother died 2 days after birth of young.
8	A	100	300	41	29	3	12	0	One mother died during parturition with litter in utero.
1	A	100	300	8	0	..	6	6	Litter was failing on 25th day. After receiving 0.5 gm. of rice bran extract daily the mother weaned the litter 9 days later.
8	A	250	600	36	18	2	14	6	
1	A	250	600	9	1	..	6	6	Litter was failing on 12th day of lactation when mother was given 1.0 gm. of rice bran extracts, daily, following which she successfully weaned her young.
6	B	250	600	29	17	2	11	0	
2	B	250	600	15	0	..	12	12	The litters of these two mothers were failing on the 10th and 17th days of lactation respectively. One gram daily of rice bran extract to mothers resulted in weaning of both litters.
1	B	250	600	8	0	..	6	6	When litter was failing on tenth day of lactation, 1.0 gm. daily of a liver extract to mother resulted in successful weaning of young.

thenate during pregnancy and 600  $\mu$ g. daily during lactation did not prevent marked failures in lactation. In the cases of three mothers failure of lactation was changed to success after the mothers were given separately from the ration and in addition to the various pure crystalline components of the B complex, 1 gm. daily of an extract from rice bran. A daily dose of only 0.5 gm. of this extract was sufficient for one mother. Failure in lactation was changed to success in the rearing of young of another mother by a supplementary daily dose of 1 gm. of liver extracts. Such observations suggested that the rice bran and liver extracts contained a hitherto unrecognized factor essential for lactation.

In order to find the most potent source of the new lactation factor, a biological assay was carried out on brewers' yeast, rice polishings, defatted wheat embryo, and dried grass, as well as the liver extracts. These food products were tested in the absence of calcium pantothenate but in the presence of the "W" factor and vitamin K. At mating there was administered a daily dose of 500 mg. of the various test products which had to be increased to 750 mg., and in some cases to 1500 mg. in order to secure successful weaning of litters (table 3). It will be noted that wherever a natural food product was used, although reproduction was abnormal in many instances, indicated by either sterilities or stillbirths, a large proportion of females in the experiments of every product tested were able to rear their young. We consider 21 to 23 days as a normal lactation period on our best stock diet, but some of the mothers on ration C (table 3) supplemented with the various test products fed at 500 to 1500 mg. daily were able to rear and wean their young only by prolonging the lactation period to 29 to 38 days. Relative to the liver extracts, it should be pointed out that these were administered from weaning up to mating as 0.2 gm. daily doses. At mating the dose was increased to 0.5 gm. daily. In two instances the daily dose had to be further increased to 1000 mg. These were given in the absence of the "W" factor, since the latter was provided in abundance by such extracts.

TABLE 8

*Series I experiments. Distribution of the new lactation factor in natural food products used as supplements (ration C).*

NUMBER OF FEMALES MATED	FOOD PRODUCT	DAILY DOSE mg.	TOTAL YOUNG BORN	NUMBER OF STILLBIRTHS	NUMBER OF STERILIZATIONS	YOUNG GIVEN TO NURSE	YOUNG REARED	LACTATION EFFICIENCY INDEX	REMARKS
6	Brewer's yeast	500 to 750	45	16	0	18	18	100	
6	Rice polishings	500	62	16	0	30	23	77	Had to increase dose to 750 mg. in one case and to 1500 mg. in another.
6	Defatted wheat embryo	500	23	0	3	18	12	66	Lactation abnormal. Had to increase to 1500 mg. for one mother. Another mother died during middle of lactation period.
6	Dried grass	500	51	17	0	29	29	100	Prolonged lactation in case of 3 mothers, i.e., 29, 31, and 35 days, respectively.
6	Liver extracts (fraction D)	500 to 1000	73	12	0	35	32	91	

It will be noted that thirty-two out of thirty-five young were successfully weaned when the liver extracts were used which gave a lactation efficiency index of 91%. The significant point about the results of these experiments is that such food products as brewers' yeast, dried bakers' yeast (Sure, '41), wheat embryo, rice polishings, rice bran, and liver extracts, all potent sources of the vitamin B complex, are effectual in supplementing thiamine, riboflavin, pyridoxine, choline chloride, nicotinic acid, pantothenic acid, and vitamins A, D, E, and K, for rearing of young of the albino rat.

The next problem was to reduce the high incidence of stillbirths. Examination of the Hubbell-Mendel-Wakeman salt

mixture no. 351 ('37) revealed that it does not furnish a sufficiency of manganese, if the reports of Daniels and Everson ('35) and Orent and McCollum ('31) are made the basis of judgment. The demonstration of the indispensability of zinc in the nutrition of the rat (Stirn, Elvehjem and Hart, '35) led Phillips and Hart ('35) to introduce this element in their salt mixture. The Ca:P ratio in the Hubbell-Mendel-Wakeman salt mixture is about 4 to 1, a ratio unsatisfactory for successful lactation according to Cox and Imboden ('36). A new salt mixture was therefore prepared consisting essentially of the Phillips and Hart mixture modified in ways believed to represent the application of available knowledge concerning the need for various inorganic nutrients. The formula finally devised is given in table 4. The product is referred to as "Sure's salts no. 1."<sup>3</sup>

TABLE 4  
*Composition of Sure's salts no. 1.*

	gm.		gm.
NaCl	335.0	KI	1.6
K <sub>2</sub> HPO <sub>4</sub> (anhydrous)	645.0	MnSO <sub>4</sub> (anhydrous)	7.0
CaHPO <sub>4</sub> ·2H <sub>2</sub> O	190.0	CuSO <sub>4</sub> (anhydrous)	0.4
MgSO <sub>4</sub> (anhydrous)	99.0	Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> K <sub>2</sub> SO <sub>4</sub>	0.4
CaCO <sub>3</sub>	600.0	NaF	0.5
Ferric citrate (powdered)	55.0	ZnCl <sub>2</sub>	0.5

### *Series II experiments*

This series of experiments were carried out on rations D, E, and F with Sure's salt mixture no. 1. In all of the series II experiments 100 µg. calcium pantothenate were given to each animal daily, 250 µg. daily during pregnancy and 600 µg. daily during lactation. A ration containing 10% of our purified casein and 10% of hot alcohol-extracted beef-blood fibrin was found to promote greater growth than either 20% casein or 20% blood fibrin; hence, the introduction of ration F. During pregnancy and lactation ration F was supplemented with 0.2%

<sup>3</sup> See footnote 2, page 501.

cystine in the diet, in order to insure a sufficiency of sulphhydryl compounds for reproduction and milk secretion (Sure, '41). Similarly, the rest of the rations were fortified with cystine only during gestation and lactation.

Comparison of tables 2 and 3 (rations A, B and C) with table 5 (rations D, E and F) shows that there was a marked improvement in reproduction on rations carrying the new salt mixture. Such comparison can only be made between control animals which received the basal diets devoid of any supplements of natural food products or extracts therefrom. Out of thirty-six animals bred on rations A and B, containing the Hubbell-Mendel-Wakeman salt mixture, there were eleven

TABLE 5

*Series II experiments. Rice bran extract as a source of the new lactation factor (Rations D, E, and F) and control experiments.*

NUMBER OF FEMALES MATED	DAILY DOSE	TOTAL YOUNG BORN	NUMBER OF STILL- BIRTHS	NUMBER OF STERILITIES	YOUNG GIVEN MOTHERS TO NURSE	YOUNG REARED	LACTATION EFFICIENCY INDEX
gm.							
8	0.25	61	0	0	45	0	0
1	0.50	8	0	..	6	0	0
1	0.5 to 1.0	12	0	..	6	4	66
9	1.0	80	0	0	52	49	94
1	1.5	13	0	..	6	6	100
.....							
20 controls		151	15	2	98	6	6

sterilities and eighty-four stillbirths out of 184 young born (table 2). On the other hand, on rations D, E, and F, containing Sure's salt mixture, out of twenty animals mated there were only two sterilities, and out of 151 young born there were fifteen stillbirths (table 5). In other words, the change to the new salt mixture produced a reduction of stillbirths from 46 to 10% and a reduction of sterilities from 30 to 10%. Lactation was, however, a marked failure even with the new salt mixture. Only one female out of twenty reared her young. Out of ninety-eight young given to rear only six were weaned, or an incidence of infant mortality of 94%. Further supple-

mentation of Sure's salts with cobalt and boron in the form of cobalt chloride and sodium tetraborate (anhydrous), respectively, in the same concentration as the zinc chloride in that salt mixture did not prevent complete failure in lactation. Out of thirty-six young given to six mothers to rear, all died within the first few days of lactation.

It is difficult at present to say what change in the salt mixture is to be credited with the greater success secured in reproduction. We incline to the view that the increase in manganese content was a determining factor. It is also possible that a change in the Ca : P ratio played an important role.

It was anticipated that the administration of 0.25 gm. of the rice bran extract daily during pregnancy would perhaps furnish a sufficiency of the new dietary essential at least during the first week of lactation; from the point of subsequent failure of young, administration of various extracts or concentrates could be begun. Such procedure, however, proved a failure (table 5). Reproduction was excellent. Out of sixty-one young born to eight mothers all were alive and virile but most of the young died on the second day of lactation. A 0.5-gm. dose was tried on only one mother but it proved a failure. When another mother on a 0.5 gm. allowance lost two of her six young on the third day, the daily dose was increased to 1.0 gm. which resulted in the successful rearing and weaning of the rest of the litter. The increases of the rice bran extract to 1.0 gm. daily resulted in the successful rearing of forty-nine out of fifty-two young given nine mothers to rear, or a lactation efficiency index of 94%. One mother reared her litter on a 1.5 gm. daily dose during gestation and lactation. The average lactation period was 23 days which is quite normal according to our experience.

Calculated from the biological assay of the rice bran extract given by the manufacturers of this product, each gram contained 107 µg. thiamine, 7 µg. riboflavin, 107 µg. pyridoxine, and 284 µg. pantothenic acid. Accordingly each female that received a supplementary dose of 1.0 gm. of the rice bran extract, was given the following daily doses of the components of the B complex: 15 µg. thiamine, 110 µg. riboflavin, 15 µg.

pyridoxine, 15 mg. choline chloride, 150 µg. calcium pantothenate and the "W" factor extract from 1.0 gm. liver extracts. It was particularly essential that the thiamine dose be adjusted, because of the possibility of encountering toxic doses for fertility and lactation (Perla, '37; Perla and Sandberg, '39; Sure, '39).

Attempts to save failing nurslings the second or third days of lactation by giving the rice bran extracts or liver extracts were unsuccessful; also attempts to provide the lactation factor later than the seventh day of pregnancy were unsuccessful. The most dependable procedure for insuring successful lactation was to provide a sufficiency of the lactation factor beginning at the time of breeding. Four females on ration F, given 1.0 gm. of liver extracts during pregnancy and lactation, had thirty-seven live young and successfully reared twenty-two out of twenty-three young in an average lactation period of 21 days. Two litters weaned by their mothers in 18 days showed accelerated growth.

#### *Is the new lactation-promoting factor organic or inorganic?*

In order to determine whether the lactation factor is organic or inorganic, the rice bran extracts<sup>4</sup> were ashed, and the ash mixed with ten times the amount of sucrose (equivalent to 1.0 gm. of the original extract) was administered daily in caster cups. That the lactation factor is not an inorganic substance became evident from the following results: Eight out of nine mothers failed in lactation. In addition, twelve females failed in lactation when receiving the daily dose of ash from liver extracts<sup>5</sup> equivalent to 1.0 to 2.0 gm. of the original extracts. It is evident then that the lactation factor must be organic in nature.

<sup>4</sup> The ash from the rice bran extracts was prepared by the Research Laboratories of Galen Company at a temperature of about 1000°C. The ash from the liver extracts was prepared by the Research Laboratories of Merck and Company at a temperature approximating 1600°C.

<sup>5</sup> See footnote 4.

*Response in lactation to inositol administration*

The addition of five to ten times the amount of the "W" factor found necessary for optimum growth did not prevent failure in rearing of young. Furthermore, Frost and Elvehjem ('37) state that the residue from the extract of the "W" factor contained very little activity. After rearing three litters successfully on the residue equivalent to 2 gm. of the original liver extracts a fuller's earth adsorption product was prepared. The new lactation factor was found in the filtrates. On the daily allowance of this concentrate equivalent to 2 gm. of liver extracts containing 320 mg. solids, five mothers reared thirty-three out of thirty-four young. The litter of one mother, however, reached maintenance on the fifteenth day of lactation and maintenance persisted for 7 days. Another litter showed loss of weight on the seventeenth day and maintenance on the succeeding 3 days.

The recent report of Ansbacher ('41) that p-aminobenzoic acid is a chromotrichia factor for the rat, and that of Woolley ('41) that inositol is an antialopecia factor for the mouse, as well as the claim of Pavcek and Baum ('41) that inositol is an "antispectacled" and growth-promoting factor for the rat, warranted trial of these substances for effect on lactation. The results with daily doses of 15 mg. p-aminobenzoic acid were negative. A daily dose of 30 mg. inositol resulted in a prompt response in the case of the first mother, i.e., a gain of 16 gm. in 24 hours and 33 gm. in 48 hours in weights of the litter, and weaning of the litter in 8 days subsequent to the inositol administration. The response to inositol therapy in the case of the second mother was similar.

*Influence on lactation of administration of p-aminobenzoic acid, in presence and absence of inositol*

It was then decided to attempt to rear nursing young of the albino rat on diets in which the B complex was supplied by only the pure chemical substances proven to date to make up

this complex. For this reason the "W" factor was withheld from the females at mating. The experiments were conducted in three series and the following daily additions to the synthetic vitamin B complex mixture were given to the mothers during pregnancy and lactation: (a) 15 mg. p-aminobenzoic acid; (b) 30 mg. inositol; (c) 15 mg. p-aminobenzoic acid and 30 mg. inositol. The results obtained to date on reproduction and lactation, respectively, are as follows: (a) Out of ninety-two young born there were only three dead, or 3.3% stillbirths; out of fifty-three young given nine mothers to rear, thirty-two were weaned. (b) Out of five litters, two were born dead; two mothers failed in lactation with litters of six each; one mother weaned five young. Per cent of stillbirths was 30. (c) Out of forty-six young born to five mothers there was only one stillbirth; out of twenty-eight young given five mothers to rear, twenty-two were weaned.

Combining the results of series 1 and 3, it is evident that out of eighty-one young allowed to nurse, fifty-four were weaned or a lactation efficiency index of 67% due to the addition of p-aminobenzoic acid, and in the absence of the "W" factor. When it is considered that in the absence of the new lactation factor and in the presence of the "W" factor, the lactation efficiency is only about 5%, the lactation-promoting properties of p-aminobenzoic acid assume marked significance. It would appear then that either p-aminobenzoic acid or a substance of similar physiological properties is a component of the new dietary factor essential for lactation.

During the past 7 years several papers have appeared by Nakahara and associates ('34, '35, '39) on the existence of "L and L<sub>1</sub>" factors essential for lactation. Since the diets of the Japanese workers contained natural foods and were deficient in more than one respect, it is impossible to evaluate the results of their findings and to make correlations of their results with those submitted in this communication.

## DISCUSSION

It is apparent from the experimental data submitted that an adequacy of the known pure crystalline components of the vitamin B complex, namely, thiamine, riboflavin, pyridoxine, and pantothenic acid, which were fed in amounts six times that required for excellent growth, did not satisfy the requirements for lactation. Choline chloride and nicotinic acid did not supply the missing factor essential for rearing of young. Suspecting that possibly various components of the B complex were being furnished in insufficient amounts during gestation, lactation-doses were given to six mothers beginning at mating, but the failure in lactation was 100%. The daily supplementary dose of the rice bran extract, which changed a 95% failure in lactation to 91% success, introduced only 8.8 mg. nitrogen, of which 1.47 mg. was ammonia, 0.45 mg. urea nitrogen, and 3.0 mg. amino acid nitrogen. It seems then that only 4 to 5 mg. was utilizable nitrogen. In view of the fact that an excellent source of amino acids was furnished by the proteins in rations A to F inclusive, and the amount of nitrogen introduced by the rice bran extract was so minute, it seems improbable that the lactation factor is a known amino acid. On the other hand, the distribution of the factor in such food products as brewers' yeast, bakers' yeast, wheat embryo, rice polishings, rice bran extract, and liver extracts, indicates that it may properly be described as associated with the vitamin B complex. I, therefore, tentatively propose to designate this new lactation factor as " $B_x$ " the "B" associating it with the "B" complex and the "x" denoting that it is as yet chemically unidentified. It is possible, of course, that the " $B_x$ " factor is composed of more than one dietary substance essential for lactation.

The marked responses obtained in lactation following p-aminobenzoic acid additions in the absence of any extracts from natural food products point to the possibility that either this substance or a substance of similar physiological proper-

ties is a component of the "B<sub>x</sub>" factor. The response obtained to inositol in lactation also suggests that this substance may be another component of the "B<sub>x</sub>" factor.

#### SUMMARY AND CONCLUSIONS

Young albino rats can exhibit excellent growth on diets in which the vitamin B complex was supplied by pure thiamine, riboflavin, pyridoxine, choline, pantothenic acid, nicotinic acid and "W" factor from liver extracts. Such diets with the amounts of these supplements greatly increased will not support either adequate reproduction or lactation. Experiments with new salt mixtures resulted in marked improvement in reproduction but lactation was still a failure. A rice bran extract and a liver extract were found to supply a factor, the presence of which resulted in 90% success in lactation. Tests with the ash of such products were negative; hence, the factor is organic. Para-aminobenzoic acid or a related compound is a component of the new factor tentatively designated "B<sub>x</sub>"; preliminary tests suggest that inositol may also be a component.

The author is indebted to Dr. David Klein of the Wilson Laboratories for supplying fraction D of liver extracts; to Parke, Davis & Co., for the 2-methyl-1-4-napthoquinone; to Armour & Co., for the beef blood fibrin; to Merck & Co., for the thiamine, riboflavin, pyridoxine, calcium pantothenate, and choline chloride; to Mr. H. A. Smith of the Galen Co., Berkeley, Cal., for the rice bran extracts; and to the Cerophyl Laboratories, Kansas City, Mo., for the dried grass.

#### LITERATURE CITED

- ANSBACHER, S. 1941 p-Aminobenzoic acid, a vitamin. *Science*, vol. 93, pp. 164-165.
- BLACK, S., D. V. FROST AND C. A. ELVEHJEM 1940 The relation of vitamin B<sub>x</sub> and pantothenic acid to factor W studies. *J. Biol. Chem.*, vol. 132, pp. 65-76.

- Cox, W. M., JR., AND M. IMBODEN 1936 The role of calcium and phosphorus in determining reproductive success. *J. Nutrition*, vol. 11, pp. 147-177.
- DANIELS, A. L., AND G. J. EVERSON 1935 The relation of manganese to congenital debility. *J. Nutrition*, vol. 9, pp. 191-204.
- FROST, E. V., AND C. A. ELVEHJEM 1937 Further studies on factor W. *J. Biol. Chem.*, vol. 121, pp. 255-273.
- HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in nutrition of chicks. *J. Biol. Chem.*, vol. 138, pp. 459-467.
- HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. *J. Nutrition*, vol. 14, pp. 273-286.
- JONES, D. B. 1931 Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins. *U. S. D. A. Circular*, No. 183, pp. 1-22.
- JONES, J. H. 1938 The use of fibrin in synthetic diets. *J. Nutrition*, vol. 15, pp. 269-276.
- NAKAHARA, W., F. INUKAI AND S. KATO 1934 A specific dietary factor for lactation. *Proc. of Imp. Academy*, vol. 10, pp. 268-271.
- NAKAHARA, W., F. INUKAI AND D. UGAMI 1935 On the nature of factor L, a specific dietary factor for lactation. *Inst. Phys. Chem. Res.*, vol. 28, pp. 154-161.
- 1939 Notes on the methods for vitamin L tests. *Inst. Phys. and Chem. Res.*, vol. 36, pp. 327-335.
- ORENT, E. R., AND E. V. MCCOLLUM 1931 Effects of deprivation of manganese in the rat. *J. Biol. Chem.*, vol. 92, pp. 651-679.
- PAVCEK, P. L., AND H. M. BAUM 1941 Inositol and spectacled eye in rats. *Science*, vol. 93, p. 502.
- PÉRLA, D. 1937 Toxic effects of an excess of B<sub>1</sub> in rats. *Proc. Soc. Exp. Biol. and Med.*, vol. 37, pp. 169-172.
- PÉRLA, D., AND M. SANDBERG 1939 Metabolic interdependence of vitamin B<sub>1</sub> and manganese. Reciprocal neutralization of their toxic effects. *Proc. Soc. Exp. Biol. and Med.*, vol. 41, pp. 522-527.
- PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. *J. Biol. Chem.*, vol. 109, pp. 657-664.
- SCHANTZ, E. J., C. A. ELVEHJEM AND E. B. HART 1940 The comparative nutritive value of butterfat and certain vegetable oils. *J. Dairy Sci.*, vol. 23, pp. 181-189.
- STIRN, F. E., C. A. ELVEHJEM AND E. B. HART 1935 The indispensability of zinc in the nutrition of the rat. *J. Biol. Chem.*, vol. 109, pp. 347-360.
- SURE, B. 1939 Influence of massive doses of vitamin B<sub>1</sub> on fertility and lactation. *J. Nutrition*, vol. 18, pp. 187-194.

- SURE, B. 1940 Quantitative requirements of the components of the vitamin B complex for lactation and growth of nursing young of the albino rat. *J. Nutrition*, vol. 19, pp. 57-70.
- 1941 The lactation-promoting properties of cystine when added to casein diets. *J. Nutrition*, vol. 22, pp. 491-498.
- TOENNIES, G. 1937 The sulfur containing amino acid methionine. *Growth*, vol. 1, pp. 337-370.
- WOOLLEY, D. W. 1941 Identification of the mouse antialopecia factor. *J. Biol. Chem.*, vol. 139, pp. 29-35.

## THE INFLUENCE OF PRENATAL DIET ON THE MOTHER AND CHILD<sup>1</sup>

J. H. EBBS, F. F. TISDALL AND W. A. SCOTT

*Departments of Paediatrics and Obstetrics, University of Toronto, Canada*

(Received for publication June 16, 1941)

During the past 25 years, the number of deaths in infants under 1 year of age has been markedly reduced. The number of deaths in the first few weeks of life, however, has been altered very little. Apart from congenital abnormalities, birth trauma and prematurity, there still remain a number of unexplainable deaths. This study was undertaken in order to determine the effect of poor and good prenatal diets upon the outcome of pregnancy and condition of infant during first months of life. Only patients who had not reached the end of the sixth month of pregnancy and those patients who signified their intention of being confined in the Toronto General Hospital were included in the study. If any major disease was found that patient was excluded.

Briefly, the method of study was as follows: (1) An analysis of the patient's food record was made at the beginning of observation; (2) this analysis was repeated 2 months later; (3) patients were classified into three groups, namely, those receiving a poor diet throughout, or having a supplemented diet, or subsisting on a good diet and receiving advice; (4) blood examinations were made for hemoglobin, vitamin C and phosphatase; (5) obstetrical rating was given each patient for (a) the prenatal period, during labor and convalescence, and with respect to (b) condition of baby at birth, (c) follow-up examination of the mother, (d) ability of mother to nurse

<sup>1</sup>Presented at the eighth annual meeting of the American Institute of Nutrition, Chicago, April 16, 1941.

infant, and (e) the whole course of pregnancy from beginning to end of observation; (6) babies were examined at 6 months and at 12 months of age, and records were kept of illnesses, general condition and eruption of teeth.

Each patient at the first interview was given a record form containing a space for each meal for 7 days. In this she was instructed to write down the kind and amount of each food eaten at each meal for 7 days. She was instructed to record weights of food, where possible; otherwise, to record in common measures, such as tablespoonful, cupful, ounces, or measure of solid foods in inches, or as large, medium or small vegetable or fruit. The patient then returned with this record and was interviewed by the dietitian. Each detail on the record was checked by means of questioning and comparison with amounts bought and amounts served at each meal to the whole family. Recipes were also discussed and methods of cooking. Social workers visited the homes of many of the patients in the Supplemented Group in order to check the consumption of the foods being sent. A trained worker visited the home of a small number of patients in each group and weighed the food after it had been estimated and recorded. Having arrived at the approximate amounts consumed, the foods were then totalled for the week under the following headings: eggs, meat (to include fish and fowl), milk, cheese, cream, butter, oil, bread (white and brown), cereals, potatoes, vegetables, cooked and raw, fruits (citrus and others), sugar, and miscellaneous. This list formed the basis for scoring the diet as "good," "fair" or "poor". A consultation was then held and with this diet record, knowledge of the family income, rent, and number of dependents, each patient was considered for further study.

If the diet was poor and the income low, each alternate patient was selected for help from a special fund.<sup>2</sup> Those who did not receive help were left on their poor diets, were given no dietary advice, and will be referred to as the Poor Diet Group. There were 120 in this group. Those who had

<sup>2</sup> This fund was provided by a Toronto business man.

been on an equally poor diet until the fourth or fifth month of pregnancy and then received extra food from us, will be referred to as the Supplemented-to-Good Diet Group. There were ninety of these. It was found that approximately one-half of the patients in the clinic were on moderately good diets and had sufficient income to provide a good diet if given advice. Advice in detail was then given. These will be referred to as the Good Diet Group. There were 170 of these studied.

As a basis for planning reasonable food requirements during pregnancy, we aimed at the following amounts of the essential foods: daily, 40 ounces of milk, 1 ounce cheese, 1 egg, an average serving of butter and meat, 2 servings of vegetables in addition to potato, one orange or one-half grapefruit, or 5 ounces of tomato juice, one-half of the cereals and bread consumed to be in whole grain form, 2 teaspoonfuls of cod liver oil or equivalent in concentrate, liver once a week, salt to be iodized, and medicinal iron to be used if indicated. Two tablespoonfuls of wheat germ daily were advised. The constitution of such an average diet is given in table 1.

TABLE 1

*Nutritive factors yielded by the recommended Good Diet used in this study.*

<i>Vitamins:</i>			
Calories	—	2400-2800	A — 6000 I.U. <sup>1</sup>
Protein	—	80-100 gm.	B <sub>1</sub> — 500-1000 I.U.
Fat	—	80-100 gm.	B <sub>2</sub> — 3.0-3.5 mg.
Carbohydrate	—	350-400 gm.	C — 50-75 mg.
Calcium	—	1.5 gm.	D — 500-1000 I.U.
Iron	—	0.020 gm.	
Iodine	—	In iodized salt	<sup>1</sup> International Units.

We advised those patients with sufficient income in the Good Diet Group to try to obtain the amounts given in table 1.

To the patients in the Supplemented-to-Good Group we sent daily, 30 ounces of milk, 1 egg and 1 orange. Once a week, we also sent two 16-ounce tins of factory canned tomatoes and  $\frac{1}{2}$  pound of cheddar cheese. At the clinic we distributed a palatable dried wheat germ which contained malt, and added

iron.<sup>3</sup> Two tablespoonfuls contained 12 mg. of iron. Viosterol capsules containing 2000 international units of vitamin D were supplied, with instructions to take one daily.<sup>4</sup> Advice in detail was given to the women in this group regarding the use of this extra food, and instruction was given in planning the remainder of the diet from the family income.

The average cost of the extra food supplied to the ninety women in the Supplemented-to-Good Diet Group for an average period of 4.7 months was \$25.00 per patient.

In order to offset any possible psychological factor due to the taking of medicine, patients not receiving supplemental food were given gelatin capsules resembling the viosterol capsules, but containing instead plain corn oil.

The additional food supplied to the patients in the Supplemented-to-Good Group gave the following daily average values: protein, 45 gm.; fat, 46 gm.; carbohydrate, 60 gm.; calories, 840; calcium, 1.45 gm.; iron, 15 mg.; vitamin C, 50-80 mg.; vitamin B<sub>1</sub>, 350-400 I.U., and vitamin D, 2000 I.U.

In order to eliminate errors in judgment and to offset the number of dietitians interviewing the patients, each diet record was later calculated for protein, fat, carbohydrate, calories, calcium and iron.<sup>5</sup> The material was arranged in such a form that information regarding the vitamin content could also be calculated.

In table 2, it will be noted that the Poor Diet Group and the Supplemented Group were equally low in every respect in the first record made at the beginning of the observation. The average figures of the diets of patients in the Good Diet Group are moderately good. In the second record, made about 4 or 6 weeks before confinement, the average of the diets in the Poor Diet Group is still low, although slightly increased over the first record. It will be noted that the figures in the Supplemented Group and the Good Diet Group have improved

<sup>3</sup> Embryon—Scientific Foods, Ltd., Toronto.

<sup>4</sup> Kindly supplied by Mead Johnson & Company.

<sup>5</sup> Figures for calculation of protein, fat, carbohydrate, calories, calcium and iron were chosen mainly from table 13 of "Applied Dietetics" by Francis Stern.

TABLE 2  
*Analysis of diets in pregnancy.<sup>1</sup>*

		PROTEIN	FAT	CH	CALS.	Ca	Fe
		gm.	gm.	gm.		gm.	mg.
First record	Poor Diet	56	66	213	1672	.537	10.7
	Supplemented-Good Diet	56	67	212	1690	.562	10.5
	Good Diet	81	95	261	2206	.886	14.2
Second record	Poor Diet	62	75	232	1837	.746	11.6
	Supplemented-Good Diet	94	111	283	2424	1.61	24.3
	Good Diet	92	113	293	2521	1.30	18.3

<sup>1</sup> All figures are per diem.

markedly, the former by supplying food, and the latter by education.

Table 3 shows the percentage of patients in each group on the basis of their protein intake. The improvement in the Supplemented and the Good Diet Groups will be noted. Sixty per cent of the patients in the Poor Diet Group were taking less than 60 gm. of protein daily. Seventy-eight per cent in the Supplemented Group were getting more than 80 gm. daily after the extra food was supplied. Advice to those in the Good Diet Group about increasing the consumption of milk,

TABLE 3  
*Percentage of patients in each group on basis of daily consumption of protein, calcium and vitamin C.*

	POOR DIET		SUPPLEMENTED-GOOD		GOOD DIET	
	1st record	2nd record	1st record	2nd record	1st record	2nd record
	%	%	%	%	%	%
<b>Protein</b>						
Less than 60 gm.	60	38	54	2	23	2
60 to 80 gm.	33	48	41	20	40	24
More than 80 gm.	7	14	.5	78	37	74
<b>Calcium</b>						
Less than 0.8 gm.	81	61	86	2	46	2
0.8 to 1.2 gm.	16	28	10	10	32	37
More than 1.2 gm.	3	11	4	88	22	61
<b>Vitamin C</b>						
Less than 25 mg.	82	69	84	2	46	34
25 to 50 mg.	17	31	15	48	46	56
More than 50 mg.	1	0	1	50	8	10

eggs, meat and cheese resulted in a substantial increase in the percentage who were taking a reasonable amount of protein. Thirty-six per cent of the Poor Diet Group were getting less than 0.4 gm. of calcium daily. Eighty-six per cent of the patients in the Supplemented Diet Group were getting less than 0.8 gm. of calcium daily, according to our estimated figures in the first record. The addition of milk and cheese to the diet of the Supplemented Group improved the second record greatly. All but 2% were getting more than 0.8 gm. Again the effect of education was noted in the Good Diet Group, in which all but 2% brought the intake of calcium above 0.8 gm. daily. The figures for vitamin C are based only upon the consumption of citrus fruits and tomatoes. They do not include any source of vitamin C in the general diet. It will be noted that the economic level of even the Good Diet Group did not allow the women to purchase more than 50 mg. of vitamin C in citrus fruits or tomatoes.

Table 4 illustrates how the poor diet in the Supplemented Group was improved as far as vitamin B<sub>1</sub> was concerned, by

TABLE 4  
*Vitamin B<sub>1</sub> content of low income diets.*

	INT. UNITS OF VITAMIN B <sub>1</sub>
Original diet —	
8 oz. potatoes .....	100
8 oz. bread — 80% white .....	54
1.5 oz. cooked oatmeal (7.5 gm. dry).....	13
10 oz. milk .....	60
2.7 oz. meat (beef six times, pork once).....	67
4 oz. vegetable .....	23
0.5 oz. egg — $\frac{1}{4}$ egg .....	7
	<hr/>
	324
Supplemented by —	
0.5 oz. wheat germ .....	100
30 oz. milk .....	180
1 egg .....	30
1 orange .....	20
4.5 oz. tomato .....	34
Additions to meat, vegetables and whole wheat bread	86
TOTAL .....	450 774

giving wheat germ, milk, egg, orange and tomato, and by changing from white bread to whole wheat bread.

The average duration of observation in the Prenatal Clinic was 4.4 months in the Poor Diet Group, 4.7 months in the Supplemented Group and 4.4 months in the Good Diet Group (table 5). The economic status of the patients in the three diet groups is shown in table 5. Three per cent of the patients in the Good Diet Group were receiving from other sources extra milk, meat, vegetables and fruits in addition to the relief ration, which allowed them to be in the group improved by education. The past obstetrical records of the multiparous

TABLE 5  
*Patients in prenatal diet study—miscellaneous information.*

	POOR DIET	SUPPLEMENTED- GOOD DIET	GOOD DIET
Number of patients	120	90	170
Average age	26	27	25
Average duration of prenatal observation	4.4 mos.	4.7 mos.	4.4 mos.
Primipara	31%	29%	46%
Percentage of families on welfare relief	44%	48%	3%
Average value of relief allowance (approximate only)	\$7.50	\$8.50	—
Average weekly income of families not on relief	\$12.02	\$10.94	\$16.94
Average number of persons per family	3.0	3.7	2.8
Average income per person	\$3.34	\$2.64	\$6.02

patients showed a much higher incidence of previous major complications in the Poor Diet and Supplemented Groups than in the Good Diet Group (table 6.) Those in the Supplemented Diet Group had experienced more miscarriages, stillbirths and premature births in previous pregnancies than the Poor Diet Group.

The obstetrician in charge of the patients in the Prenatal Clinic and in the Hospital has given his rating of the condition and progress of the patient in each period of pregnancy. He was unaware of the diet group to which each patient belonged. A "good" or "fair" rating indicated that in his opinion the patient had progressed satisfactorily or with minor compli-

TABLE 6  
*Observations related to pregnancy.*

	POOR DIET	SUPPLEMENTED- GOOD DIET	GOOD DIET
<b>Past obstetrical history of multiparous patients (% of cases):</b>			
Abortions	13.1	4.7	9.0
Miscarriages	38.1	39.0	24.4
Prematures	10.7	20.3	13.3
Stillbirths	9.5	4.7	2.2
<b>Obstetrician's rating during pregnancy (% of cases):</b>			
Prenatal period	Good-Fair Poor-Bad	64 36	91 9
Labor	Good-Fair Poor-Bad	76 24	97 3
Convalescence	Good-Fair Poor-Bad	88 12	96 4
Whole course of pregnancy	Good-Fair Poor-Bad	66 34	94 6
<b>Complications during pregnancy (% of cases):</b>			
Anemia		28.6	16.1
Preeclampsia		5.0	5.7
Toxemia		7.6	3.4
Hemorrhage—prenatal		5.9	5.7
Threatened miscarriage		8.4	1.1
Miscarriage		6.0	0
Premature birth		8.0	2.2
Stillbirth		3.4	0
Hemorrhage (during labor)		11.2	10.3
Endometritis		9.0	3.4
Mastitis		4.5	2.3
Breast abscess		3.0	1.1
<b>Studies of blood during pregnancy (all values are averages):</b>			
Hemoglobin at term	11.5 gm.	12.1 gm.	11.9 gm.
Ascorbic acid at term	0.47 mg. %	0.73 mg. %	0.62 mg. %
Ascorbic acid cord blood	1.0 mg. %	1.4 mg. %	1.3 mg. %
Phosphatase at term	16.5 units	14.5 units	16.6 units

cations only. "Poor" or "bad" meant that many or major complications had arisen. The rating during the prenatal period, during the actual labor, and during the 2 weeks of convalescence in the hospital is shown in table 6. The obstetrician's rating of the whole course of pregnancy from the time that the patient first came under observation in the Prenatal Clinic until she was seen 6 weeks after the birth of her baby is also presented in table 6. The mothers in the Supplemented-to-Good Diet Group proved to be better obstetrical risks. The average duration of labor was 5 hours shorter in this group than in the Poor Diet Group. We noted a marked improvement in the general mental attitude of the patients in the Supplemented Group; many of them lost their minor aches and pains, and no longer had numerous complaints.

During the prenatal period there were more cases of anemia, toxemia, and threatened miscarriage in the Poor Diet Group, while the total number of complications in this group was almost double that in the Supplemented-Good Group (table 6). The complications which affected the rating during labor in the Poor Diet Group were chiefly 6% of miscarriages, 8% of premature births, and 3.4% of stillbirths, while in the Supplemented Good Group there were only 2.2% of prematures and no miscarriages or stillbirths (table 6). After delivery there were fewer cases of uterine or breast infections in the Supplemented-Good Group (table 6).

The effect of prenatal diet is reflected in the average levels of hemoglobin, vitamin C and phosphatase in the blood of the mother at the time of delivery (table 6). The average amount of hemoglobin at the time of delivery was slightly higher in the Supplemented-Good Group. The average level of ascorbic acid in the mother's blood at term and in the cord blood was proportional to the vitamin C obtained by consumption of citrus fruit and tomatoes. Phosphatase is an enzyme which has to do with the laying down of new bone. Phosphatase is increased when there are difficult or abnormal conditions in bone formation. Thus we have found that the phosphatase of the mother's blood is more than double the average values

from the sixth month to term when twins are present (Ebbs and Scott, '40). The phosphatase in the mother's blood was appreciably lower in the Supplemented Group than in the other two groups. This became apparent from the seventh month onward after the Supplemented Group had been receiving viosterol capsules, while the other two groups had not received a source of vitamin D. This seemed to indicate that expectant mothers receiving vitamin D and an adequate diet were better able to provide for new bone in the developing fetus.

The average birth weight of the babies born of mothers in the Poor Diet Group was 7 pounds 10 ounces; in the Supplemented Group, 7 pounds 7 ounces; and in the Good Diet Group 7 pounds 6½ ounces. The additional calories do not appear to have influenced the size of the baby.

The relation of prenatal diet to the incidence of breast feeding is shown in table 7.

TABLE 7  
*Observations related to infancy.*

CATEGORIES OF INTEREST	POOR DIET	SUPPLEMENTED-GOOD DIET	GOOD DIET
<i>Breast feeding in relation to prenatal diet (% of cases):</i>			
In hospital			
Breast feeding	81	95	88
Artificial feeding	19	5	12
Six weeks after birth			
Breast feeding	59	86	71
Artificial feeding	41	14	29
<i>Principal illnesses in babies during first 6 months (% of cases):</i>			
Frequent colds	21.0	4.7	4.7
Bronchitis	4.2	1.5	5.7
Pneumonia	5.5	1.5	0.9
Rickets	5.5	0	0.9
Tetany	4.2	0	0
Dystrophy	7.0	1.5	0
Anemia	25.0	9.4	17.1
Deaths	3	0	0

An attempt is being made to follow the progress of the babies born of the mothers in this study to determine the influence, if any, of prenatal diet upon the future condition of the baby. These observations are not completed, but a brief summary can be given of the first 250 babies followed to the age of 6 months (table 7). The increased incidence of minor and major diseases in the babies born of mothers in the Poor Diet Group is quite striking. The general condition of the babies in the Supplemented and Good Diet Groups was on the whole much better. In a large proportion one could tell the diet group of the mother by looking at her baby. Two of the three infant deaths in the Poor Diet Group resulted from pneumonia, and the other from prematurity.

#### SUMMARY

The prenatal diets of 400 women with low incomes were studied. One group found to be on a poor diet was left as a control, a second group on a poor diet was improved by supplying food during the last 3 or 4 months of pregnancy, and a third group, found to have moderately good prenatal diets was improved by education alone.

During the whole course of pregnancy the mothers on a good or supplemented diet enjoyed better health, had fewer complications and proved to be better obstetrical risks than those left on poor prenatal diets.

The incidence of miscarriages, stillbirths and premature births in the women on poor diets was much increased.

The incidence of illness in the babies up to the age of 6 months and the number of deaths resulting from these illnesses were many times greater in the Poor Diet Group.

#### CONCLUSION

While it is recognized that there are other important factors in the successful outcome of pregnancy, this study suggests that the nutrition of the mother during the prenatal period

influences to a considerable degree the whole course of pregnancy, and in addition directly affects the health of the child during the first 6 months of life.

#### ACKNOWLEDGMENTS

We wish to thank the many persons who have helped in this study. Particularly we are indebted to Miss Marjorie Bell, Miss Helen Kelly, Miss Elizabeth Castle, Miss Nina Johnstone, and the obstetrical staff and nursing staff of the Toronto General Hospital.

The diet analyses and diet instruction were personally supervised by Miss Winnifred Moyle, with the assistance of her dietetic staff at the Toronto General Hospital.

#### LITERATURE CITED

- EBBS, J. H., AND W. A. SCOTT 1940 Blood phosphatase in pregnancy an indication of twins. *Am. J. Obstetrics & Gynecology*, vol. 39, pp. 1043-1044.

# THIAMINE, NICOTINIC ACID, RIBOFLAVIN AND PANTOTHENIC ACID IN RYE AND ITS MILLED PRODUCTS

AARON J. IHDE AND HENRY A. SCHUETTE

*Department of Chemistry, University of Wisconsin, Madison*

(Received for publication July 1, 1941)

It has been generally agreed that the cereal grains, especially the germ and bran portions, are rich sources of some of the vitamins of the B complex. In the case of wheat, considerable data have been presented to show that this is true; data for the other cereal grains are meager. Although rye flour ranks in importance second only to wheat flour, little is known regarding its vitamin content, and even less regarding the distribution of the vitamins throughout the kernel.

Baker and Wright ('35), using a bradycardia technique, reported 22 µg. per gram of thiamine in rye germ. Scheunert and Schieblich ('37), using a rat-feeding method, found 9 µg. per gram of thiamine in rye germ and 3 µg. per gram in the whole rye. Booher and Hartzler ('39), also using a rat growth technique, reported 3.9 and 4.7 µg. per gram of thiamine in two samples of whole rye. Lunde, Kringstad and Olsen ('39) reported 4.2 µg. of thiamine in whole rye meal and 1.6 µg. per gram in rye flour as determined by the thiochrome method. Schultz, Atkin and Frey ('41) tested ten samples of rye for thiamine by their fermentation method ('37) and found a range of 4.0–5.7 µg. per gram with 4.84 as an average figure.

Scheunert and Schieblich ('37), using the Bourquin-Sherman method ('31), found 2.4 µg. per gram of riboflavin in the whole rye and 10 µg. per gram in rye germ. Lunde, Kringstad and Olsen ('39) reported 10 µg. per gram in rye germ. Lunde,

Kringstad and Olsen ('39) reported 1.6 µg. per gram of riboflavin in whole rye meal and 0.6 µg. per gram in rye flour of 60% extraction, as determined by a fluorescence method. Kringstad and Naess ('39), using a cyanogen bromide-aniline method, reported 13 µg. per gram of nicotinic acid in whole rye. Recently Snell and Wright ('41) have reported 58 to 63 µg. per gram in a sample of rye flour as determined by their microbiological method. No assays for pantothenic acid or vitamin B<sub>6</sub> (pyridoxine) are reported to date.

#### EXPERIMENTAL PART

Analyses for thiamine, nicotinic acid, riboflavin and pantothenic acid were made on representative products<sup>1</sup> of the rye-milling process, namely, whole rye meal, rye germ, middlings, dark flour, and white flour. The middlings and flours were obtained in duplicate series, one ground from whole rye, the other from rye which had been degerminated. The latter series was representative of common milling practice, since the germ is usually removed by a so-called "smutting" process in order to improve the keeping quality of the flour. The white flour samples represent a separation of the straight flour in such manner that the coarse, colored particles are diverted into a dark flour, leaving the "white" fraction largely free of the outer, colored portions of the kernel. Two samples of white flour from whole rye differed in that one was bleached while the other was not so treated. The sample of white flour from degerminated rye was also bleached.

Preliminary assays for thiamine were made by the colorimetric method of Melnick and Field ('39) as modified by Emmett, Peacock and Brown ('40). Satisfactory results were obtained for rye germ (9.3 µg. per gram) and for middlings (3.4 µg. per gram), but concentration difficulties led to the abandonment of the procedure in favor of the method of Henry ('41) which utilizes the growth-response of *Phycomyces blakesleeanus* to thiamine and its phosphorylated esters as a

<sup>1</sup> Acknowledgment is made to Frank H. Blodgett, Inc., of Janesville, Wisconsin, who furnished the materials used in this investigation.

method for measuring the small amounts present in plant tissues.

The chemical methods for nicotinic acid, based on color-formation with cyanogen bromide and an aromatic amine, have been shown by Kodicek ('40) and Waisman and Elvehjem ('41) to give erroneous results with cereal products. Therefore, the microbiological method of Snell and Wright ('41), based upon lactic acid production by *Lactobacillus arabinosus* 17-5, was used.<sup>2</sup>

Riboflavin was determined by the microbiological method of Snell and Strong ('39) using *Lactobacillus casei* ε. Pantothenic acid was determined by the method of Pennington, Snell and Williams ('40) using the same organism.

TABLE 1

*Thiamine, nicotinic acid, riboflavin and pantothenic acid in rye products.*

PRODUCT	THIAMINE μg./g.	NICOTINIC ACID μg./g.	RIBOFLAVIN μg./g.	PANTOTHENIC ACID μg./g.
Whole rye	2.4	12.9	1.5	10.4
Rye germ	9.3	27.0	4.46	13.9
Middlings from whole rye	3.3	16.7	2.5	23.1
Middlings from degermanated rye	3.3	17.7	2.0	23.1
Dark flour from whole rye	3.2	12.2	1.7	18.4
Dark flour from degermanated rye	3.6	12.5	1.8	14.9
White flour from whole rye (not bleached)	1.5	7.1	0.76	7.1
White flour from whole rye (bleached)	1.6	7.3	0.69	7.25
White flour from degerminated rye (bleached)	1.4	7.3	0.68	6.5

In all cases the assays were made on no less than three individual samples of differing weight. In case of doubtful results the assays were repeated until consistent results were obtained. Standard curves were prepared with every set of assays. Recovery experiments were also made on every run with recoveries usually ranging between 90 and 105%. The results obtained, expressed in micrograms per gram, are summarized in table 1.

<sup>2</sup> We wish to thank Prof. F. M. Strong of the Biochemistry Department of the College of Agriculture for his interest and suggestions and Mr. Lester Teply of his laboratory for making the nicotinic acid determinations.

## DISCUSSION

The data in table 1 have been averaged and treated in table 2 so as to show what percentages of thiamine, nicotinic acid, riboflavin, and pantothenic acid in the whole grain are diverted into the various products of the milling process.

Although the germ contains nearly four times as much thiamine as the whole grain, it contributes only 6% of the total thiamine of the grain since it constitutes such a small proportion of the kernel.<sup>3</sup> The middlings, representing in large part the covering of the seed, contain one-fourth of the

TABLE 2  
*Distribution of thiamine, nicotinic acid, riboflavin and pantothenic acid in rye products.*

	RYE	THIAMINE	NICOTINIC ACID	RIBOFLAVIN	PANTOTHENIC ACID
Whole rye	100				
Germ	1.6	6.1	4.1	5.5	1.9
Middlings	18	24.6	29.2	31.1	35.4
Flour, straight	80.4	69.2	66.7	63.4	62.7
Flour, dark	24.4	34.4	28.8	32.8	29.3
Flour, white	56	34.8	38.0	30.5	33.4

total thiamine while the straight flour contains approximately two-thirds. Further refining of the straight flour results in a concentration of one-half of this thiamine in the dark portion which constitutes only one-third of the total flour.

In spite of this refining loss, white rye flour appears to be a richer source of thiamine than white wheat flour. The value of 1.5 µg. per gram for straight wheat flour reported by Schultz, Atkin and Frey ('39) is equal to the value found for white rye flour during this study. All other recent investigators, however, report lower values for wheat flour. Booher and Hartzler ('39) reported 0.92 µg. per gram, using a rat growth method. Scheunert and Schieblich ('37) report a

<sup>3</sup> Experimental dissections and weighings were made on four different samples of rye grain. The germ content varied between 1.38 and 1.78% with 1.61% as an average figure.

value of 1  $\mu\text{g}$ . per gram, using a similar method. Lunde, Kringstad and Olsen ('39), using a thiochrome method, found 0.9  $\mu\text{g}$ . per gram in straight wheat flour as compared to 1.6  $\mu\text{g}$ . per gram in straight rye flour. Values reported for patent wheat flour are even lower, ranging from 0.5  $\mu\text{g}$ . per gram by Booher and Hartzler ('39) to 0.7  $\mu\text{g}$ . per gram by Schultz, Atkin and Frey ('39).

The lower refining losses in rye flour as compared to wheat flour are further borne out by comparison with the data of Schultz, Atkin and Frey ('39). These workers showed that the straight flour, representing 72% of the wheat kernel, contains only 18% of thiamine present in the original wheat.

It is thus evident that differences in the structure of the rye kernel and in the technique of rye-milling result in a flour nutritionally superior to straight wheat flour insofar as thiamine is concerned. It must also be remembered that white rye flour is seldom marketed as such but is blended with varying amounts of the dark rye flour which further serves to enrich the product.

The distribution of nicotinic acid and riboflavin roughly parallels that of thiamine although rye, like the other cereal grains, cannot be considered a rich source of these vitamins. Pantothenic acid does not appear to be concentrated to any degree in the germ portion, but the middlings are a rich source and the dark flour contains a significant amount.

Reference to table 1 shows that there is no significant difference between the products milled from whole rye and from degerminated rye. The germ constitutes such a small portion of the whole kernel that its removal prior to milling results in only a slight loss of the total vitamins.

Bleaching of the white flour appears to result in no destruction of vitamins except possibly in the case of riboflavin. Slightly lower values for this vitamin were always noticed in bleached samples as compared to unbleached ones. The differences, however, were hardly great enough to be considered conclusive.

## SUMMARY

Samples of the products of the rye-milling process were assayed for thiamine, nicotinic acid, riboflavin and pantothenic acid by microbiological methods.

Except for pantothenic acid, the concentration of each of the vitamins is greatest in the germ. The middlings are also a potent source of each of the vitamins but particularly of pantothenic acid.

The flour contains approximately two-thirds of the total vitamins in the whole grain. It is, therefore, a definitely richer source of the vitamin B complex than white wheat flour. Even a high degree of refining fails to bring the thiamine content to the low level characteristic of patent wheat flour.

Rye flour contains approximately one-half of its total vitamins in the dark portion which constitutes only one-third of the total flour.

Degermination of the rye prior to milling fails to remove enough of the vitamins to be detectable by the methods used since the germ constitutes such a small portion of the whole kernel.

Bleaching appears to cause no vitamin losses except possibly in the case of riboflavin.

## LITERATURE CITED

- BAKER, AUDREY Z., AND MARGARET D. WRIGHT 1935 The vitamin B<sub>1</sub> content of foods. *Biochem. J.*, vol. 29, p. 1802.
- BOOHER, LELA E., AND EVA R. HARTZLER 1939 The vitamin B<sub>1</sub> content of foods in terms of crystalline thiamine. *U. S. Dept. Agr., Tech. Bull.*, 707.
- BOURQUIN, ANNE, AND H. C. SHERMAN 1931 Quantitative determination of vitamin G (B<sub>2</sub>). *J. Am. Chem. Soc.*, vol. 53, p. 3501.
- EMMETT, A. D., GAIL PEACOCK AND R. A. BROWN 1940 Chemical determination of thiamine by a modification of the Melnick-Field method. *J. Biol. Chem.*, vol. 135, p. 131.
- HENRY, B. W. 1941 The relation of vitamin B<sub>1</sub> to crown-gall development. Ph.D. thesis, University of Wisconsin.
- KODICEK, E. 1940 Estimation of nicotinic acid in animal tissues, blood and certain foodstuffs. I. Method. II. Applications. *Biochem. J.*, vol. 34, pp. 712, 724.
- KRINGSTAD, H., AND T. NAESS 1939 Eine colorimetrische Methode zur Bestimmung von Nicotinsäure und Nicotinsäureamide in Nahrungsmitteln. *Z. physiol. Chem.*, vol. 260, p. 108.

- LUNDE, G., H. KRINGSTAD AND A. OLSEN 1939 The amount of vitamin B<sub>1</sub> and B<sub>2</sub> (riboflavin) in ordinary Norwegian cereals and bread. *Nord. Med.*, vol. 3, p. 2533; *Chem. Abst.*, vol. 34, p. 531 (1940).
- MELNICK, D., AND H. FIELD, JR. 1939 Chemical determination of vitamin B<sub>1</sub>. I. Reaction between thiamine in pure aqueous solution and diazotized *p*-amino acetophenone. II. Method for estimation of the thiamine content of biological materials with the diazotized *p*-amino acetophenone reagent. III. Quantitative enzymic conversion of cocarboxylase (thiamine pyrophosphate) to the free vitamin. *J. Biol. Chem.*, vol. 127, p. 505; p. 515; p. 531.
- PENNINGTON, D., E. E. SNELL AND R. J. WILLIAMS 1940 An assay method for pantothenic acid. *J. Biol. Chem.*, vol. 135, p. 213.
- SCHEUNERT, A., AND M. SCHIEBLICH 1937 Über den Vitamingehalt von Weizen und Roggen und der daraus hergestellten Mehle und Brot. *Biochem. Z.*, vol. 290, p. 398.
- SCHULTZ, A. S., L. ATKIN AND C. N. FREY 1937 A fermentation test for vitamin B<sub>1</sub>. *J. Am. Chem. Soc.*, vol. 59, p. 2457.
- 1939 The vitamin B<sub>1</sub> content of wheat, flour and bread. *Cereal Chem.*, vol. 16, p. 643.
- 1941 A preliminary survey of the vitamin B<sub>1</sub> content of American cereals. *Cereal Chem.*, vol. 18, p. 106.
- SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. *Ind. Eng. Chem., Anal. Ed.*, vol. 11, p. 346.
- SNELL, E. E., AND L. D. WRIGHT 1941 A microbiological method for the determination of nicotinic acid. *J. Biol. Chem.*, vol. 139, p. 675.
- WAISMAN, H. A., AND C. A. ELVEHJEM 1941 Chemical estimation of nicotinic acid and vitamin B<sub>6</sub>. *Ind. Eng. Chem., Anal. Ed.*, vol. 13, p. 221.



## NICOTINIC ACID CONTENT OF MEAT AND MEAT PRODUCTS<sup>1</sup>

J. M. MCINTIRE, HARRY A. WAISMAN, LAVELL M. HENDERSON AND  
C. A. ELVEHJEM

*Department of Biochemistry, College of Agriculture,  
University of Wisconsin, Madison*

(Received for publication August 4, 1941)

The nicotinic acid potency of a number of animal tissues was reported in a previous paper (Waisman et al., '40). The results were based on the growth responses in dogs maintained on the modified Goldberger diet. By careful standardization of the animal with the pure vitamin it was possible to obtain a fairly reliable figure for the antipellagra activity of the various tissues. At the time these determinations were made certain limitations in this method were recognized, but it was decided that the animal assay was more reliable than the chemical methods which were available at that time. Much time has been spent during the past 2 years in perfecting the cyanogen bromide method in order to apply it to animal tissue assays. The reports of Melnick and Field ('40), Kodicek ('40), and Bandier ('40) have been especially valuable in helping to perfect our procedure.

### EXPERIMENTAL AND RESULTS

The dried meats which were available for the chemical determination of nicotinic acid were those previously assayed for nicotinic acid by the biological method (Waisman et al., '40),

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by a grant from the National Live Stock and Meat Board made through the National Research Council.

as well as additional samples which were assayed previously for thiamine (Mickelsen et al., '39 a), riboflavin (Mickelsen et al., '39 b), pantothenic acid (Waisman et al., '39) and pyridoxine (Henderson et al., '41). The procedure used was essentially that of Melnick and Field ('40) with the slight modifications described by Waisman and Elvehjem ('41 a). In the majority of cases the recovery of the added nicotinic acid was in the range of 90 to 110%.

The results are summarized in table 1. The figures represent the results of over two hundred analyses on seventy different samples. These samples are listed in the approximate order of potency.

The organ tissues, liver and kidney, were found to be the most potent sources of nicotinic acid. The livers of beef, pork, and lamb have the same range of potency, namely, 15.1 to 22.7 mg. per 100 gm. of fresh tissue. This range compares favorably with the 18.0 mg. given by Kringstad and Naess ('38) and the figures of Karrer and Keller ('39) namely 11.8 and 9.3 mg. per 100 gm. of fresh tissue, respectively. Unlike liver, the kidney tissues of beef and pork differ to some extent in nicotinic acid content. The range for beef kidney was 6.4 to 8.3 mg., which is similar to that given by Bandier ('39), but it is considerably lower than the figure of 19.4 mg. per 100 gm. of fresh tissue given by Karrer and Keller. The range of pork kidney was found to be 9.0 to 10.5 mg., which is higher than the figure of 6.5 mg. per 100 gm. of fresh tissue given by Bandier for kidney. Values obtained from assaying the muscular tissue of various animals were in good agreement with one another ranging from 5.6 to 9.1 mg. This range is somewhat higher than the figures reported by Bandier ('39), Kringstad and Naess ('38), and Karrer and Keller ('39) who gave values of 4.73, 4.9 and 3.8 mg. per 100 gm. of fresh muscle tissue, respectively. Our assays on heart, spleen and lung give somewhat higher values than those reported by the other workers. The nicotinic acid content of light and dark chicken meat was found to be about the same. This is interesting since the assays of thiamine, riboflavin, and pantothenic acid have

TABLE 1  
Nicotinic acid values obtained by chemical analyses of animal tissues.

Tissue	Sample Number	Milligrams of Nicotinic Acid per 100 gm. of Tissue		Tissue	Sample Number	Milligrams of Nicotinic Acid per 100 gm. of Tissue	
		Dry weight	Fresh weight			Dry weight	Fresh weight
Beef liver	98	69.1	21.0	Fried veal steak	45	28.9	8.4
Fried beef liver	121	53.3	15.8	Veal hindquarter	103	33.0	8.9
Beef liver	131	77.8	22.7	Veal hindquarter	129	36.4	9.1
Beef liver	150	58.6	16.1	Veal hindquarter	134	31.4	6.8
Beef liver	152	51.2	15.1	Lamb leg	80	32.7	8.5
Veal liver	70	50.8	13.2	Pork ham	32	20.3	5.6
Veal liver	97	69.5	20.2	Fried pork ham	52	14.4	3.7
Veal liver	158	72.1	19.5	Pork ham	95	27.0	6.6
Pork liver	122	94.5	29.8	Pork ham	124	35.0	8.3
Pork liver	153	60.4	18.7	Pork ham	139	33.0	8.4
Pork liver	159	71.4	20.0	Smoked ham	47	25.2	8.2
Lamb liver	96	59.1	17.2	Fried smoked ham	48	30.2	14.3 <sup>1</sup>
				Smoked ham	102	19.5	5.6
Beef kidney	81	36.2	8.3	Smoked ham	116	13.1	3.9
Beef kidney	126	32.1	6.4	Tender ham	72	14.0	4.4
Stewed beef kidney	112	14.7	5.2	Tender ham	117	15.3	5.2
Pork kidney	62	47.9	10.5	Tender ham	119	23.0	7.7
Pork kidney	83	45.4	9.9	Boiled ham	115	19.3	6.7
Pork kidney	137	45.0	9.1	Boiled ham	101	18.3	6.0
Stewed beef heart	54	15.9	3.3	Pork loin	36	21.2	5.6
Stewed beef heart	87	24.4	7.3	Broiled pork loin	37	20.8	4.5
Beef heart	133	42.4	8.2	Fried pork loin	38	23.2	5.6
Beef heart	143	37.0	7.0	Pork loin	74	21.4	6.1
Veal heart	155	50.7	10.6	Pork loin	125	22.2	6.1
Beef spleen	76	23.3	6.2 <sup>1</sup>	Pork loin	156	22.2	6.7
Stewed beef spleen	109	21.1	6.3 <sup>1</sup>				
Beef spleen	130	38.8	8.2	Beef pancreas	64	18.0	5.8
				Beef tongue	82	22.0	6.1
Fried beef round	42	34.1	9.1	Beef brain	132	23.2	5.1
Beef muscle	105	21.5	6.4	Beef brain	144	22.2	4.7
Roast round of beef	107	20.0	4.3	Beef lung	138	30.4	6.2
Beef round	128	37.0	8.7	Light chicken	68	29.7	7.3
Beef round	145	30.2	7.7	Dark chicken	66	23.8	6.1
Broiled beef round	147	22.5	...	Cod muscle	135	13.5	2.3
Fried beef round	148	19.2	...	Salmon muscle	136	26.4	7.4
Beef round	151	33.0	8.2				
Beef round	154	27.8	6.9				
Beef round	157	27.9	7.8				

<sup>1</sup> Calculated approximation.

shown the dark meat to be the more potent vitamin source (Waisman and Elvehjem, '41 b). The muscle of salmon is a richer source of nicotinic acid than cod muscle, the values being 7.4 and 2.3 mg., respectively. Since we were interested in the nicotinic acid content of meat as prepared for consumption, we assayed various samples of home-cooked and commercially-prepared meats. The analysis of fried beef liver, fried beef muscle and fried pork muscle showed very little loss during the cooking process. Assays on stewed beef heart, kidney and spleen showed losses as high as 50% of the vitamin during cooking. The results of analyses of broiled and roasted beef muscle indicate that some nicotinic acid is lost during the cooking process. Commercially-prepared products such as boiled ham, smoked ham and tenderized ham were also analyzed for their nicotinic acid content. All showed some loss due to the processing. The results of these assays are in close agreement with the biological assays.

#### DISCUSSION

Many of the samples used for this work had been assayed previously by the biological method, and it was of special interest to compare the results obtained by the two methods. The two organ tissues, liver and kidney, found to be the most potent sources of nicotinic acid by biologic assay on the dog were also found to be the most potent source by the chemical method. However, the values obtained from the two assays varied to some extent. The chemical method gave an average value of 18 mg. per 100 gm. of fresh liver tissue, but the biological assay gave a value of 25 mg. Values obtained from the assays of fresh kidney were in similar disagreement. On the other hand, the two methods gave values for muscular tissue and less potent organ tissues that were in close agreement. It appears from such results that growth responses obtained in the biological assays, especially on liver and kidney tissue, are not due entirely to the nicotinic acid content of the meat but to other factors present in the meat which are required by the dog and are not present in sufficient amounts in the basal ration.

The results of our assays have quite closely paralleled those of the other workers or have been somewhat higher. We feel that such variation is due both to methods of extraction and analysis used by the various workers and to the source of meat. It is known that variation in the diet may affect the vitamin content of the tissue.

The loss of some of the vitamin potency in cooking is not likely to be due to the destruction of nicotinic acid since it is a very stable compound. However, being water soluble, it is probably leached from the meat during the processing and remains in the cooking water.

#### SUMMARY

Nicotinic acid content of meats was determined by the cyanogen bromide-aniline method.

Liver and kidney were the richest sources of nicotinic acid. The average value for liver was 18 mg. per 100 gm. of fresh tissue. All animal tissue contains a significant amount of nicotinic acid.

Cooking and commercial processing caused some loss of the vitamin.

#### LITERATURE CITED

- BANDIER, E. 1939 Quantitative estimation of nicotinic acid in biological material. *Biochem. J.*, vol. 33, p. 1130.  
— 1940 On nicotinic acid—especially methods for its quantitative estimation in organic material. Copenhagen, Einar Munksgaard.  
HENDERSON, L. M., H. A. WAISMAN AND C. A. ELVEHJEM 1941 Distribution of pyridoxine (vitamin B<sub>6</sub>) in meat and meat products. *J. Nutrition*, vol. 21, p. 589.  
KARRER, P., AND H. KELLER 1939 Bestimmung von nicotinsaureamid in tierischen organen. *Helv. Chim. Acta*, vol. 22, p. 1292.  
KRINGSTAD, H., AND T. NAESS 1938 Über die kolorimetrische bestimmung von nicotinsäure und nicotinsaureamid. *Naturwissenschaft*, vol. 26, p. 709.  
KODICEK, E. 1940 Estimation of nicotinic acid in animal tissues, blood and certain foodstuffs. I. Method, II. Applications. *Biochem. J.*, vol. 34, pp. 712, 724.  
MELNICK, D., AND H. FIELD, JR. 1940 Determination of nicotinic acid in biological materials by means of photoelectric colorimetry. *J. Biol. Chem.*, vol. 134, p. 1.

- MICKELSEN, O., H. A. WAISMAN AND C. A. ELVEHJEM 1939 a The distribution of vitamin B<sub>1</sub> (thiamin) in meat and meat products. *J. Nutrition*, vol. 17, p. 269.
- 1939 b The distribution of riboflavin in meat and meat products. *J. Nutrition*, vol. 18, p. 517.
- WAISMAN, H. A., O. MICKELSEN AND C. A. ELVEHJEM 1939 The distribution of the chick antidermatitis factor (pantothenic acid) in meat and meat products. *J. Nutrition*, vol. 18, p. 247.
- WAISMAN, H. A., O. MICKELSEN, J. M. MCKIBBIN AND C. A. ELVEHJEM 1940 Nicotinic acid potency of food materials and certain chemical compounds. *J. Nutrition*, vol. 19, p. 483.
- WAISMAN, H. A., AND C. A. ELVEHJEM 1941 a Chemical estimation of nicotinic acid and vitamin B<sub>6</sub>. *Ind. and Eng. Chem., Anal. Ed.*, vol. 13, p. 221.
- 1941 b The vitamin content of meat. Burgess Publishing Co., Minneapolis, Minnesota.

# THE UTILIZATION BY CALVES OF THE ENERGY CONTAINED IN BALANCED RATIONS COMPOSED OF COMBINATIONS OF DIFFERENT FEEDS

H. H. MITCHELL AND T. S. HAMILTON

WITH THE TECHNICAL ASSISTANCE OF W. T. HAINES

*Animal Nutrition Division, University of Illinois, Urbana*

(Received for publication July 14, 1941)

The investigation to be reported in this paper was undertaken to test with growing calves the proposition that, although the energy wastages of rations in digestion, aside from the associative effects of one feed upon the digestibility of another, are characteristics of the various feeds of which they are composed, as expressed in their chemical compositions, the energy wastages of rations in metabolism are not characteristic functions of the constituent feeds but rather are functions of the combinations of digestible nutrients supplied to the tissues of the body. To the extent that these combinations satisfy the nutritive requirements of the tissues, the metabolizable energy of the digestible nutrients will be the more perfectly utilized and less will be wasted. If the mixture of digestible nutrients is adequate for the maximum performance of all tissue functions, then the utilization of metabolizable energy will be maximal. If it is inadequate in respect to one or more essential nutrients, eventually the wastage of metabolizable energy will increase, and conceivably the metabolizable energy may be completely wasted as animal heat (specific dynamic effect). This eventuality may be deferred until the body's stores of the lacking nutrients become exhausted; and in some animals bred through generations to fatten readily, a further deferment may result from the ready transformation

of organic nutrients into body fat, irrespective of their balanced character (Mitchell, Hamilton and Haines, '40).

The rational basis for this proposition (Mitchell, '34) and its historical development and evidential support (Mitchell, '37) have been presented by the senior author, while the junior author has reported the results of an investigation illustrating clearly its validity in rodent nutrition (Hamilton, '39).

If the above proposition is correct, animal feeds (and human foods also) do not possess distinctive net energy values, separately determinable and additive in estimating the net energy contained in rations or diets. Their contents of metabolizable energy are more or less characteristic, but the net availability of the metabolizable energy depends upon the nutritive adequacy of the metabolizable nutrients provided by the rations of which the feeds are components. Hence, the metabolizable energy in rations equally well-balanced with reference to the nutritive requirements of the test animal, should be equally well utilized, regardless of the number or kinds of feeds combined in the rations. This particular aspect of the problem was the object of the experiments described below.

#### PLAN OF THE EXPERIMENTS

Four rations were designed (1) to contain similar proportions of the various classes of nutrients as determined by proximate chemical analysis, (2) to contain combinations of different feeds, no one feed occurring in more than one ration, and (3) to be adequate in all essential nutrients.

The main constituents of the rations and their proximate chemical composition are given in table 1. All rations were supplemented with 56 gm. of steamed bone meal and 10 gm. of a fortified cod liver oil<sup>1</sup> per head daily, while rations A and B were supplemented in addition with 100 gm. per head daily of a dried yeast.<sup>2</sup>

<sup>1</sup> Nopeo XX.

<sup>2</sup> Northwestern.

The utilization of the gross energy in each ration was determined upon four grade Shorthorn calves. Rations A and B were tested in 1938-1939 on one group of four calves, and rations C and D in the following year on another group of four calves.

The tests involved either two or three determinations of the fasting heat production of the calves, carried out on the fourth and fifth days of fast if the values for the 2 days agreed

TABLE 1  
*Proximate chemical composition of the experimental rations.*

RATION	DRY MATTER	CRUDE PROTEIN	ETHERIC EXTRACT <sup>1</sup>	ASH <sup>1</sup>	CRUDE FIBER <sup>1</sup>	NITROGEN FREE EXTRACT <sup>1</sup>	GROSS ENERGY
	%	%	%	%	%	%	cal./gm.
Ration A: alfalfa hay 30, corn 40, linseed meal 30	90.54	18.71	4.24	4.41	9.30	53.87	4.156
Ration B: timothy hay 30, barley 50, meat scraps 20	91.67	19.87	2.98	9.12	12.91	47.90	4.089
Ration C: red clover hay 15, oats 60, cottonseed meal 25	91.51	18.61	5.01	4.72	13.08	49.36	4.141
Ration D: oat straw 20, wheat 45, soybean oil meal 20, whole pressed cottonseed 15	91.47	20.83	3.28	3.86	13.18	50.80	4.086

<sup>1</sup> Computed from average analyses taken from Morrison's "Feeds and Feeding."

within 2%, or on the fourth, fifth and sixth days in the unusual event that such agreement was not attained. The averages thus secured, expressed to the W<sup>.75</sup> kg., served as bases for the estimation of the heat increments of feeding for the various test rations. The validity of this procedure, in the opinion of the writers, has not been successfully challenged and, in fact, may be justified on practical grounds regardless of the existence or non-existence of a functional minimum level of metabolism less than the fasting metabolism by a quantity of heat originating from the specific dynamic action of the body nutrients undergoing metabolism (Forbes and Swift, '41).

The utilization of energy in the four test rations was carried out in a metabolism period extending over 12 to 14 days (preceded by a preliminary period of at least 7 days), in which the gross energy in feed, feces, and urine was determined, and in a 2- or 3-day period in the respiration chamber, in which the heat production was determined from the daily consumption of oxygen and the daily production of carbon dioxide and methane. The respiration experiment was terminated after 2 days if the values per 24 hours agreed within 2%. In most cases, the determination of heat production followed the digestion and metabolism test, and in those cases where this was not true, it was preceded by a preliminary feeding period of at least 7 days. Each ration was tested at two planes of nutrition, one estimated to approximate energy equilibrium and the other supermaintenance.

The schedule of the experiments, indicating merely the order in which the fasting heat production and the various ration tests were carried out, together with the average body weights of the steers while in the respiration chamber, is given in table 2. An attempt was made to avoid possible age effects by testing each ration in one order with two steers and in the reverse order with the other two steers. The high and the low planes of nutrition for each ration were also tested in different orders for the same reason.

The steers were confined in metabolism stalls maintained at a temperature of 75°F. and at a humidity of approximately 50% and were fed twice daily. They were weighed and watered each morning before feeding and watered again before the second feeding. The heat production was determined in an open-circuit respiration chamber previously described (Mitchell et al., '32).

All rations during each period, and composite samples of all collections of feces and urine, were analyzed for nitrogen and their heats of combustion (gross energy) measured in an adiabatic Parr oxygen-bomb calorimeter. The dry matter content of all rations was also determined.

All calorimetric calculations were made on the basis of daily heat productions corrected to a standard day of 12 hours standing and 12 hours lying down, following the procedure introduced by Armsby.

TABLE 2  
*Experimental schedule for the 2 years of work.*

PERIOD	STEER 37-1		STEER 37-2		STEER 37-3		STEER 37-4	
	Ration and plane of nutrition <sup>1</sup>	Body weight	Ration and plane of nutrition <sup>1</sup>	Body weight	Ration and plane of nutrition <sup>1</sup>	Body weight	Ration and plane of nutrition <sup>1</sup>	Body weight
		kg.		kg.		kg.		kg.
1	A—H	316	A—L	271	B—H	319	B—L	307
2	Fast	300	Fast	268	Fast	306	Fast	290
3	A—L	306	A—H	326	B—L	295	B—H	359
4	Fast	295	B—H	341	A—L	304	A—H	383
5	B—L	303	Fast	333	Fast	294	Fast	382
6	B—H	347	B—L	329	A—H	334	A—L	368
7	Fast	331	Fast	318	Fast	325	Fast	369
	STEER 40-1		STEER 40-2		STEER 40-3		STEER 40-4	
1	Fast	238	Fast	231	Fast	254	Fast	219
2	C—L	241	C—L	235	D—L	265	D—L	228
3	C—H	277	C—H	269	D—H	296	D—H	256
4	D—H	303	D—H	295	C—H	312	C—H	267
5	D—L	294	D—L	275	C—L	304	C—L	260
6	Fast	283	Fast	274	Fast	306	Fast	260

<sup>1</sup> L = low plane; H = higher plane.

#### EXPERIMENTAL RESULTS

The average contents of metabolizable energy per kilogram of dry matter and the average apparent digestibility of nitrogen for the four experimental rations at the two levels of feeding are presented in table 3. In respect to these items, the rations did not differ greatly, but for reasons that are not at all evident, the methane production on rations C and D, expressed as the gross energy of the methane produced per kilogram of dry matter in the ration, was much greater than that on rations A and B, indicating a more active paunch fermentation for the former rations. It should also be noted that rations A and B were more readily consumed than rations C and D, and in particular that higher levels could be fed because of their greater palatability. Also, ration A proved to be more palatable than ration B.

The fasting heat production of the steers, expressed in calories per  $W_{kg}^{75}$ , is given in table 4. The average value for each steer has been used in computing heat increments of feed for the experimental rations tested. The average values for all

TABLE 3  
*Metabolizability of energy and digestibility of nitrogen of the experimental rations. Averages for four steers.*

RATION	GROSS ENERGY CONSUMED	METABOLIZABLE ENERGY		METHANE ENERGY PER KILOGRAM OF DRY MATTER	APPARENT DIGESTIBILITY OF NITROGEN
		Per kilogram of dry matter	Percentage of gross energy		
	cal.	cal.	%	cal.	%
		At lower plane of nutrition			
A	14184	2886	63.54	400	76.63
B	12040	2650	59.92	422	81.06
C	11382	2408	52.92	722	74.26
D	11499	2471	55.25	763	79.32
		At higher plane of nutrition			
A	27654	2911	63.97	303	72.64
B	23206	2785	62.63	370	78.82
C	21568	2419	54.17	606	72.55
D	20873	2512	56.86	644	77.12

TABLE 4  
*The fasting heat production of the steers, expressed in calories per  $W_{kg}^{75}$ .*

STEER NO.	TEST	FASTING HEAT PRODUCTION	STEER NO.	TEST	FASTING HEAT PRODUCTION
37-1	1	86.93	40-1	1	94.82
	2	79.36		2	90.90
	3	77.66		Average	92.86
	Average	81.32			
37-2	1	69.17	40-2	1	77.74
	2	78.97		2	74.16
	3	71.70		Average	75.95
	Average	73.28			
37-3	1	72.16	40-3	1	82.01
	2	69.57		2	75.02
	3	77.22		Average	78.52
	Average	72.98			
37-4	1	69.08	40-4	1	86.75
	2	76.36		2	75.88
	3	70.13		Average	81.32
	Average	71.86			

steers was 78.51 cal. per kilogram of W.<sup>75</sup>. This value is higher than the average recently obtained (Mitchell, Hamilton and Haines, '40) for four somewhat lighter steer calves, i.e., 71.75 cal.

The most significant energy metabolism data secured in the respiration chamber with reference to the four experimental rations and the two planes of nutrition are summarized in table 5. The percentage net availability of the metabolizable energy was quite similar for the four experimental rations at the lower plane of nutrition and also at the higher plane of nutrition. In accordance with previous findings with reference to the effect of the plane of nutrition on the utilization of food energy (Forbes et al., '28, '30; Wiegner and Ghoneim, '30; Mitchell et al., '32), the net availability of metabolizable energy at the higher plane of nutrition was lower than that at the lower plane.<sup>3</sup>

As the values in the last column of table 5 reveal, the plane of nutrition varied considerably among the experiments in which the different rations were tested, even within the two series of experiments at so-called "low" and "high" planes of nutrition. A more exact comparison of the utilization of metabolizable energy in the four rations would be afforded if computations could be made for the point of energy equilibrium, and for levels above this point, computing heat increments from the heat production at an exact maintenance level of feeding.

This may be done by fitting the following equation to the eight sets of data secured for each experimental ration:

$$m = ax + by \quad (1)$$

in which  $m$  is the total intake of metabolizable energy,  $a$  is the estimated basal heat production at the prevailing body weight,  $b$  is the energy balance if positive, and  $x$  and  $y$  are the amounts of metabolizable energy expressed in calories presumably

<sup>3</sup> The average percentage difference in heat production between successive days in the calorimeter, for the feeding tests as well as the fasting tests, was 1.49, and in 73% of the tests it was less than 2.

TABLE 5  
*The utilization of metabolizable energy of the experimental rations.*

NATION	STEER	AT THE LOWER PLANE OF NUTRITION				AT THE HIGHER PLANE OF NUTRITION													
		Estimated basal heat production	Metabolizable energy consumed	Heat increment <sup>1</sup>	Fraction of metabolizable energy	Plane of nutrition <sup>2</sup>	Estimated basal heat production	Metabolizable energy consumed	Fraction of metabolizable energy	Net energy	Total	Heat increment <sup>1</sup>	Fraction of metabolizable energy	Net energy	Total	Heat increment <sup>1</sup>	Fraction of metabolizable energy	%	%
A	37-1	59.53	94.06	214.0	cal.	72.66	77.2	122	%	6093	20995	6850	14145	67.4	232				
	37-2	48.93	91.71	302.7	cal.	61.44	67.0	126		5617	16023	6204	9819	61.3	175				
	37-3	53.13	74.39	200.3	cal.	54.86	73.1	102		5635	16130	5844	10286	63.8	181				
	37-4	60.40	99.97	293.2	cal.	70.65	70.7	117		6224	17790	5875	11915	67.0	191				
	Average					72.0													64.9
	B	37-1	58.98	71.82	cal.	122.3	59.59	83.0	101	6531	15979	5740	10239	64.1	157				
B	37-2	56.54	72.86	195.4	cal.	53.82	73.2	94		5816	14061	5144	8917	63.4	153				
	37-3	51.99	69.04	221.9	cal.	40.85	67.9	90		5559	13074	4317	8757	67.0	159				
	37-4	52.72	72.34	249.6	cal.	47.38	65.5	90		5924	15087	5268	9769	65.0	165				
	Average					72.4													64.9
	C	40-1	56.77	57.75	164.5	cal.	4130	71.5	73		6307	11481	3935	7546	65.7	120			
	40-2	45.51	51.77	164.7	cal.	3530	68.2	78		5048	11231	4244	6987	62.2	138				
C	40-3	57.14	70.64	206.4	cal.	5000	70.8	87		5833	13217	5329	7888	59.7	135				
	40-4	52.67	61.08	145.8	cal.	4650	76.1	88		5365	10842	4485	6357	58.6	119				
	Average					71.7													61.6
	D	40-1	65.85	72.04	167.3	cal.	5531	76.8	84		6745	12642	3897	.8745	69.2	130			
	40-2	51.33	70.97	246.0	cal.	4657	65.3	90		5269	12273	5057	7216	58.8	137				
	40-3	51.56	59.19	228.1	cal.	3658	61.5	71		5606	11821	5064	6757	57.2	121				
D	40-4	47.74	52.42	164.7	cal.	3595	68.6	75		5209	10758	4927	5801	54.1	111				
	Average					68.0													59.8

<sup>1</sup> The difference between the observed heat production and the estimated basal heat production at the prevailing body weight.

<sup>2</sup> The net energy intake expressed as a percentage of the estimated basal heat production at the prevailing body weight.

required per calorie of basal heat (maintenance) and per calorie of energy balance (body increase), respectively. This equation expresses a purely conventional picture of the energy metabolism, a picture possessing certain pragmatic advantages in animal nutrition but questionable rational or evidential support (Mitchell, '27). Its pragmatic value depends upon the fact that the relationship between the heat production of an animal and its food consumption is curvilinear at low levels of intake, but at or near the point of energy equilibrium it is rectilinear, or approximately so, for a considerable range of food intake above maintenance (Brody and Procter, '33).

In the above equation, the value of  $x$  can be assumed to be constant only for planes of nutrition above maintenance. When the energy balance is negative,  $x$  would tend to decrease, because of the better utilization of feed energy in sub-maintenance nutrition. Hence, before fitting the above equation to the four sets of data for the four experimental rations, a correction for this fact had to be made in those cases in which the energy balance was negative.

First, the general nature of the relationship between the utilization of metabolizable energy,  $\frac{Q_n}{Q_m} \times 100$ , and the plane of the nutrition,  $Q_p$ , as measured by the net energy intake expressed as a percentage of the basal heat production, was determined by fitting to all of the data for all of the rations an equation of the type found to be suitable for this purpose by Brody and Procter ('33). Using the method of least squares in this procedure, the following equation resulted:

$$\frac{Q_n}{Q_m} \times 100 = 77.00 e^{-0.001166 Q_p} \quad (2)$$

According to this equation, at energy equilibrium,  $Q_p = 100$ , the net availability of metabolizable energy,  $\frac{Q_n}{Q_m} \times 100$ , equals 68.32. In correcting the value of  $m$  (equation 1) for any set of data for which the plane of nutrition is less than 100, the plane of nutrition,  $Q_p$ , is substituted in equation 2 and the equation is solved for  $\frac{Q_n}{Q_m} \times 100$ . Then the observed percentage utilization of the metabolizable energy (table 5) is lowered

by an amount equal to the difference between 68.32 and the value of  $\frac{Q_n}{Q_m} \times 100$  thus found. Applying this percentage to the basal heat production of the steer gives the corrected *m* value.

To the observed data of metabolizable energy, basal heat production and energy balance, corrected as explained above where correction was needed, for each experimental ration, equation 1 was fitted by the method of least squares, with the results shown in the second and third columns of table 6. The

TABLE 6

*Estimated net availability of metabolizable energy for maintenance and for body increase.*

RATION	NET AVAILABILITY OF METABOLIZABLE ENERGY			
	Unadjusted estimates		Estimates adjusted to constant rate of paunch fermentation <sup>1</sup>	
	Maintenance	Body increase	Maintenance	Body increase
A	% 72.5	% 60.3	% 72.6	% 59.5
B	72.3	55.1	72.8	54.4
C	69.6	44.3	71.2	44.2
D	64.4	53.7	66.0	53.3
Average	69.68	53.34	70.67	52.84

<sup>1</sup> Four hundred calories produced as methane per kilogram of dry ration.

net availability of metabolizable energy for maintenance is equal to  $100 \div x$ , while that for body increase is equal to  $100 \div y$ .

It will be noted that the percentage utilizability of metabolizable energy, for both maintenance and body increase, is lower for rations C and D than for rations A and B. This might possibly be a result of the greater paunch fermentation per kilogram of dry matter consumed observed with rations C and D than with rations A and B (table 3). A greater production of methane is presumably associated with a greater heat of fermentation, which, while actually non-metabolizable, is of necessity included in the metabolizable energy. Hence, the metabolizable energy in all tests and all animals was adjusted to a constant methane production, equivalent to 400 methane calories per kilogram of dry matter consumed, on the basis of an assumed ratio (Krogh and Schmit-Jensen,

'20) of 50 cal. heat of fermentation per gram mol of methane. The results secured by fitting equation 1 to these adjusted values are given in columns 4 and 5 of table 6, expressed as  $100 \div x$  and  $100 \div y$ , respectively.

The adjusted estimates, somewhat more than the unadjusted estimates, reveal a close similarity in the utilization of the metabolizable energy of the four experimental rations, especially for maintenance. The one discordant value in the general picture presented by the data of table 6, is the low value for body increase secured with ration C. In this connection it may be pointed out that the estimated net availabilities for body increase are probably less accurate than those for maintenance, because most of the metabolizable energy was used for maintenance. This is particularly true for rations C and D at the higher levels of feeding, at which only 11 to 38% of the net energy consumed was stored in the body.

The data of table 6 thus testify strongly to the similar utilization of the metabolizable energy of the four experimental rations, composed of combinations of different feeds but presumably equally well balanced.

#### SUMMARY AND CONCLUSIONS

It has been shown in metabolism and respiration experiments on eight steer calves of predominantly Shorthorn breeding that the metabolizable energy in four specially designed experimental rations is approximately equally well utilized for maintenance and for body increase. These rations were composed of combinations of different feeds, no one feed occurring in more than one ration, and were so designed both to contain similar proportions of the various classes of nutrients distinguished by proximate chemical analysis, and to be adequate in all essential nutrients.

The similarity in the net availability of the metabolizable energy of rations so constructed lends support to the hypothesis that the extent to which metabolizable energy is utilized for maintenance and tissue synthesis is not a function of the particular feeds included in the ration, but is dependent,

immediately or eventually, on the adequacy of the combination of digestible nutrients thus presented to the tissues in covering their demands for nutriment.

The authors wish to acknowledge gratefully the assistance of other members of the Division of Animal Nutrition in carrying out these arduous and painstaking experiments, particularly the assistance of Dr. F. I. Nakamura in the analysis of the chamber air; and of Mr. Frank Simpson in the care of the experimental animals.

#### LITERATURE CITED

- BRODY, S., AND R. C. PROCTER 1933 Influence of the plane of nutrition on the utilizability of feeding stuffs. Review of literature and graphic analyses of published data on the net energy and specific dynamic action problems. Mo. Agr. Exp. Sta. Res. Bul. 193. 48 pp.
- FORBES, E. B., W. W. BRAMAN AND M. KRISS, WITH THE COLLABORATION OF OTHERS 1928 The energy metabolism of cattle in relation to the plane of nutrition. J. Agr. Res., vol. 37, pp. 253-300.
- \_\_\_\_ 1930 Further studies of the energy metabolism of cattle in relation to the plane of nutrition. J. Agr. Res., vol. 40, pp. 37-78.
- FORBES, E. B., AND R. W. SWIFT 1941 The minimum base value of heat production in animals. Science, vol. 93, pp. 623-624.
- HAMILTON, T. S. 1939 The heat increments of diets balanced and unbalanced with respect to protein. J. Nutrition, vol. 17, pp. 583-599.
- KROGH, A., AND H. O. SCHMIT-JENSEN 1920 The fermentation of cellulose in the paunch of the ox and its significance in metabolism experiments. Biochem. J., vol. 14, pp. 686-696.
- MITCHELL, H. H. 1927 Does the net energy value of food depend upon the purpose for which it is used in the body? Science, vol. 66, pp. 289-292.
- \_\_\_\_ 1934 Balanced diets, net energy values and specific dynamic effects. Science, vol. 80, pp. 558-561.
- \_\_\_\_ 1937 The importance of the relations between energy, protein and minerals in measuring the nutritive value of feeds and rations. Proc. Am. Soc. Anim. Production, 30th Ann. Meeting, pp. 29-42.
- MITCHELL, H. H., T. S. HAMILTON, F. J. MCCLURE, W. T. HAINES, J. R. BEADLES AND H. P. MORRIS 1932 The effect of the amount of feed consumed by cattle on the utilization of its energy content. J. Agr. Res., vol. 45, pp. 163-191.
- MITCHELL, H. H., AND T. S. HAMILTON, WITH THE TECHNICAL ASSISTANCE OF W. T. HAINES 1940 The utilization by calves of energy in rations containing different percentages of protein and in glucose supplements. J. Agr. Res., vol. 61, pp. 847-864.
- WIEGNER, G., AND A. GHONEIM 1930 Über die Formulierung der Futterwirkung. Ein Beitrag zur Theorie der Verwertung des Unternährungs- und Produktionsfutters auf Grund von neuen Futterungsversuchen. Tierernährung, vol. 2, pp. 193-232.

## STUDIES ON NUTRITIONAL ACHROMOTRICHIA IN RATS

KLAUS UNNA, GRACE V. RICHARDS AND W. L. SAMPSON

*Merck Institute for Therapeutic Research, Rahway, New Jersey*

THREE FIGURES

(Received for publication July 16, 1941)

Greying of the fur in black rats maintained on diets deficient in the B complex was first observed by Morgan, Cook and Davison ('38) who showed that this condition could be corrected by the addition of "filtrate factor." Later, Lunde and Kringstad ('39) reported on the efficacy of brewers' yeast, and György, Poling and Subbarow ('40) on the value of liver extracts in curing this condition. With the identification of pantothenic acid as an important constituent of the "filtrate factor," speculation arose as to the effect of this substance on achromotrichia.

The effect of calcium pantothenate on the cure or prevention of greying of the fur in rats maintained on diets free from pantothenic acid has been reported from several laboratories (György and Poling, '40; Unna and Sampson, '40; Emerson and Evans, '41; Unna, '41; Elvehjem, Henderson, Black and Nielsen, '41). On the other hand, R. R. Williams ('40) was unable to demonstrate any effect of pantothenic acid on grey hair in rats, and Frost, Moore and Dann ('41) reported that only few of their animals could be protected from greying by pantothenic acid alone, whereas liver concentrates were much more effective. Recently, Ansbacher ('41) re-

ported greying of the fur in black rats maintained on purified diets supplemented with 500 µg. of calcium pantothenate per rat per day, and that this greying is cured by the addition of para-aminobenzoic acid.

In this communication, data are presented dealing with the effect of calcium pantothenate, liver, rice bran and other substances associated with the vitamin B complex on the achromotrichia in young rats maintained on diets free from pantothenic acid.

The animals used in these experiments were black or piebald rats originating from the Sprague Dawley strain. At 3 weeks of age the rats were placed on a diet having the following percentage composition: dextrose, 68; casein (vitamin free), 18; hydrogenated vegetable oil,<sup>1</sup> 8; salt mixture, U.S.P. XI, no. 1, 4; cod liver oil, 2; supplemented with 0.8 mg. each of thiamine, riboflavin and pyridoxine, 10 mg. of nicotinamide and 100 mg. of choline chloride per 100 gm. of diet. In part of the experiments the dextrose was substituted by sucrose; the hydrogenated vegetable oil by butterfat; and the vitamins of the B complex were added at only half the amount mentioned above. None of these variations produced any significant change in the incidence of achromotrichia.

On this regimen the rats grew at the rate of approximately 1.5 gm. per day throughout the first 3 weeks at which time their weight became stationary and greying of the fur became evident. Simultaneously with the gradual appearance of depigmented hair, the animals developed a scant coarse fur with rusty spots, inflammation of the nose, "blood-caked" whiskers and hemorrhages into the adrenals. These lesions were the same as those occurring in albino rats on a similar diet (Unna, '40 a). The process of greying frequently developed in symmetrical patterns over the body; the hair on the head usually stayed black. Only 50% of the animals survived long enough to develop an iron or silver grey fur extending over the entire body.

<sup>1</sup> Crisco.

*Effectiveness of pantothenic acid as compared with liver and rice bran in preventing achromotrichia*

In these experiments 3-week-old rats placed on the basal diet received by stomach tube daily supplements of calcium pantothenate in graded quantities ranging from 10 to 100 µg. The growth rate obtained with 25, 40 and 100 µg., as shown in figure 1 agrees closely with the growth responses previously

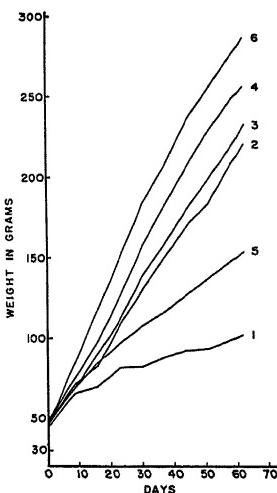


Fig. 1 Effect of daily feeding of calcium pantothenate on the growth of black rats maintained on a diet free from pantothenic acid: 1, no pantothenic acid; 2, 25 µg. of calcium pantothenate; 3, 40 µg. of calcium pantothenate; 4, 100 µg. of calcium pantothenate; 5, 50 mg. of whole dried liver; 6, 250 mg. of whole dried liver.

reported for albino rats (Unna, '40 a). Adrenal hemorrhages which on gross examination were found in approximately 50% of the rats receiving no pantothenic acid, were also observed in rats receiving 10 µg. of calcium pantothenate daily, but not encountered in those receiving 25 µg. As will be noted in figure 1, the growth response to a daily supplement of 250 mg. of whole dried beef liver<sup>2</sup> (containing 75 µg. of pantothenic acid by microbiological assay) was significantly greater than that obtained with 100 µg. of pantothenic acid.

<sup>2</sup> Kindly prepared by Dr. J. C. Keresztesy.

Figure 2 demonstrates the condition of the fur of rats maintained for 25 days on the basal diet supplemented with graded doses of calcium pantothenate or of whole dried beef liver. Rats receiving a daily supplement of 10, 25, and 40 µg. of calcium pantothenate respectively developed a marked greying of the fur, whereas those receiving 100 µg. remained black except for a few scattered grey hairs. A daily supplement of 50 mg. of dried liver, equivalent to 15 µg. of pantothenic acid,

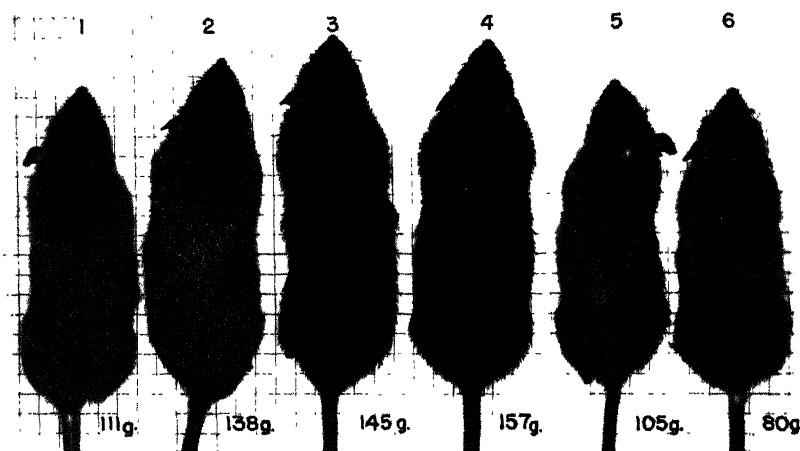


Fig. 2 Condition of the fur of rats maintained for 25 days on the basal diet free from pantothenic acid supplemented with: 1, 25 µg. of calcium pantothenate; 2, 40 µg. of calcium pantothenate; 3, 100 µg. of calcium pantothenate; 4, 250 mg. whole dried beef liver; 5, 50 mg. of whole dried beef liver; 6, no supplement.

did not prevent the achromotrichia, whereas 250 mg., equivalent to about 75 µg. of pantothenic acid, produced an effect equal to that of 100 µg. of calcium pantothenate (fig. 2).

The optimum dose for the prevention of achromotrichia was found to be approximately 100 µg. of calcium pantothenate. Increase of the daily supplement to 250 µg., 500 µg., 1 mg. or 10 mg., did not result in a further improvement in the color of the hair nor in a further gain in weight. Likewise, increasing the liver supplement to 800 mg. produced no improvement over the 250 mg. level.

Rats receiving a daily supplement of 100 µg. of calcium pantothenate have been maintained on the basal diet for more than a year. They remained black throughout the experiment. Litters obtained from these rats were also maintained on the same regimen, and for the most part, the rats of the second and third generation showed a normal black pelt, although in a few instances some scattered grey hairs were observable.

In another series of experiments, the basal diet was supplemented with 10% rice bran (containing 32 µg. of pantothenic acid per gram by microbiological assay). Achromotrichia was regularly obtained on this regimen which, on the basis of a daily food consumption of 10 gm., supplied the rats with a daily amount of about 30 µg. of pantothenic acid. Further supplements of either 50 µg. of calcium pantothenate, 0.2 gm. of dried liver or 0.5 cc. of a rice bran concentrate<sup>3</sup> prevented the achromotrichia.

#### *Curative effect of pantothenic acid as compared with liver*

Rats which had been rendered grey after 4 to 7 weeks on the basal diet, were fed daily with calcium pantothenate in amounts from 10 to 500 µg. or with whole dried beef liver. The weight response to the administration of 100 µg. of calcium pantothenate was immediate and continuous. The effect on depigmentation was somewhat delayed. Almost no change in the color of the fur could be noticed during the first week. During the second week, together with a conspicuous improvement in the quality of the hair, new black hair became noticeable, mostly in patterns on the back, on the neck and especially at the root of the tail. The process of restoration of black hair, thereafter, progressed rapidly and was usually complete by the end of the fifth week (fig. 3). Smaller doses of pantothenic acid (25 or 40 µg), although producing an appreciable weight response, did not effect cures of the achromotrichia over a period of 5 to 7 weeks, but occasionally after 8 weeks some scattered black hairs were observed and if the

<sup>3</sup> Nopeo.

rats were continued on these supplements for 15 weeks, appreciable darkening of the pelt was noticeable.

The pigmentation of the fur following the administration of whole dried beef liver in amounts of 250 mg. was neither accelerated nor superior to that obtained with calcium pantothenate, although the increase in weight was significantly greater than that following 100 µg. of calcium pantothenate (140 gm. instead of 80 gm. over a 5-week period).

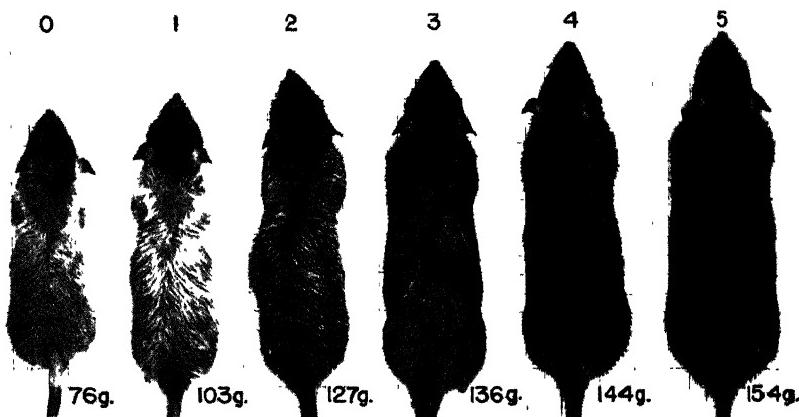


Fig. 3 Effect of daily administration of 100 µg. of calcium pantothenate on achromotrichia: (0) maintained for 22 days on the basal diet before treatment began; (1-5) same rat after 1, 2, 3, 4 and 5 weeks of treatment with pantothenic acid.

#### *Effect of biotin, inositol and para-aminobenzoic acid*

It is recognized that the basal diet used in this study is deficient in several factors recently associated with the vitamin B complex other than pantothenic acid. The effect of some of these substances now available in pure form or as concentrates has been studied in rats maintained on the basal diet.

Biotin in the form of the concentrate<sup>4</sup> practically free from pantothenic acid was administered in amounts equal to 0.5 µg.

<sup>4</sup> Concentrate no. 200 (S.M.A. Corp., Chagrin Falls, Ohio); 1 cc. containing 20 µg. of biotin according to microbiological assay.

per day without producing any improvement in achromotrichia or growth. When 100 µg. of calcium pantothenate were given simultaneously with 0.5 µg. of biotin, achromotrichia was prevented; the growth response obtained was not greater than that with pantothenic acid.

In other experiments, the biotin concentrate was administered in the same daily doses after pronounced greying had developed. No effect either on achromotrichia or on growth was observed. Furthermore, the daily feeding of biotin concentrate (equivalent to 0.5 µg.) plus 100 µg. of calcium pantothenate to grey rats did not produce an effect superior to that obtained with calcium pantothenate alone.

Inositol given in daily doses of 3 mg. did not exert any demonstrable effect on rats maintained on the basal diet. When added from the beginning of the dietary regimen, it failed to prevent the achromotrichia. Also, it was ineffective when administered in curative experiments to rats rendered grey on the basal diet.

Para-aminobenzoic acid was administered in daily doses of 3 mg. to rats either from the beginning of the dietary regimen or after they had been rendered grey. No effect was observed in either case; the appearance of grey fur and the growth rate were the same as those of the control rats. Daily administration of 3 mg. of para-aminobenzoic acid over periods extending to 6 weeks were unsuccessful<sup>5</sup> in influencing the scattered grey hairs which frequently persist following the addition of pantothenic acid and which gave these rats a "pepper and salt" appearance.

#### DISCUSSION

Our results obtained from approximately 2000 black and piebald rats show that young black rats maintained on a highly purified diet free from vitamin B complex but supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine and choline regularly develop conspicuous greying of the fur

<sup>5</sup> Since we failed to obtain greying of the fur in rats receiving adequate amounts of pantothenic acid we were unable to duplicate the report of Ansbacher ('41).

within 3 to 7 weeks. The achromotrichia is associated with the syndrome of pantothenic acid deficiency previously described for albino rats on a similar diet (Unna, '40 a). The occurrence of achromotrichia is not prevented by the addition of calcium pantothenate in doses of 10 to 40 µg. However, a daily supplement of 100 µg. of calcium pantothenate, representing approximately the optimum daily amount for growth on the basal ration described on page 554, prevents the occurrence of grey hair and restores within 4 to 6 weeks the black pigmentation of the fur in rats which have been rendered grey on the deficient diet. Frequently some scattered grey hairs persist in spite of an ample supplement of pantothenic acid. These results are in agreement with previous findings (Unna and Sampson, '40; György and Poling, '40) recently confirmed by Emerson and Evans ('41) and Elvehjem, Henderson, Black and Nielsen ('41). Williams ('40) using a basal diet differing from ours mostly in its low fat content, failed to cure greying of the fur with pantothenic acid. We have conducted experiments using a diet practically free from fat consisting of dextrose, 78%; casein (vitamin free), 18%; salt mixture U.S.P. XI no. 1, 4%; supplemented with 0.8 mg. each of thiamine, riboflavin, and pyridoxine, 10 mg. of nicotinamide and 100 mg. of choline per 100 gm. of diet; in addition the rats received a daily supplement of 100 mg. of linoleic acid concentrate and twice weekly β-carotine (50 µg.) and vitamin D (1000 U.S.P. units) dissolved in 0.15 cc. of ethyl laurate. In this experiment calcium pantothenate was found to be effective in preventing or curing grey hair occurring on this ration, although the growth of young rats on such a diet is poor, and the weight response obtained with pantothenic acid much less marked than in rats maintained on rations containing 10% fat. In our experiments as well as those of Elvehjem et al. ('41), graded responses of repigmentation with graded doses of pantothenic acid were observed, whereas Frost, Moore and Dann ('41) have reported the same incidence of partial cures with 25 µg. as well as with 200 µg. of calcium pantothenate. Also these investigators reported

more pronounced effects on achromotrichia from liver concentrates than from calcium pantothenate.

Black rats have been reared throughout three generations on the basal ration with a daily supplement of 100 µg. of pantothenic acid. No significant achromotrichia has been observed in the offspring. This successful mating of black rats confirms previous findings of Jukes ('40) and of our laboratory on albino rats (Unna, '40 b), recently confirmed by Richardson, Hogan, Long and Itschner ('41).

Biotin, inositol and para-aminobenzoic acid were found ineffective in preventing or curing the achromotrichia in rats maintained on a diet free from pantothenic acid. Emerson and Evans ('41) have likewise reported on the inefficiency of inositol. When pantothenic acid is present in the diet, these substances do not exert an additional effect beyond that of pantothenic acid alone.

Experiments with whole dried beef liver, rice bran and rice bran concentrate on a large number of black rats on our basal ration have failed to demonstrate an anti-grey hair effect of these substances sufficiently superior to that of pantothenic acid to permit postulating the existence of a separate "anti-grey hair factor" (Nielsen, Oleson and Elvehjem, '40; Morgan and Simms, '40). Furthermore, the effectiveness of liver concentrate in restoring pigmentation was destroyed after treatment with alkali. However, the growth-promoting effect of whole dried beef liver was invariably greater than that of equivalent amounts of pantothenic acid.

Pantothenic acid appears to be essential in correcting the depigmentation of the fur occurring on vitamin B complex deficient diets. Since greying is not confined to vitamin B complex deficiencies, but occurs in other conditions such as mineral deficiencies, substances other than pantothenic acid may well be effective under different conditions.

#### CONCLUSIONS

1. Black and piebald rats maintained on a B complex free diet supplemented with thiamine, riboflavin, nicotinic acid,

pyridoxine and choline develop greying of the fur within 3 to 7 weeks simultaneously with retardation in growth and the appearance of a scant coarse fur, inflammation of the nose, "blood-caked" whiskers and adrenal hemorrhages.

2. Addition of 100 µg. of calcium pantothenate prevents the development of the achromotrichia together with the other deficiency lesions, and restores within 4 to 6 weeks the black pigmentation of the fur in rats which have become grey on the basal diet.

3. The efficacy of liver and rice bran in preventing or curing achromotrichia parallels their content of pantothenic acid, although these materials exert a growth-promoting effect superior to that of pantothenic acid.

4. Black rats maintained on a B complex free diet supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, choline and pantothenic acid have been raised through three generations.

#### LITERATURE CITED

- ANSBACHER, S. 1941 Para-aminobenzoic acid—a vitamin. *Science*, vol. 93, p. 164.
- ELVEHJEM, C. A., L. M. HENDERSON, S. BLACK AND E. NIELSEN 1941 Synthetic calcium pantothenate in the nutrition of the rat. *J. Biol. Chem.*, vol. 140, p. xxxvi.
- EMERSON, G. A., AND H. M. EVANS 1941 Growth and greying of rats with total "filtrate factor" and with pantothenic acid. *Proc. Soc. Exp. Biol. and Med.*, vol. 46, p. 655.
- FROST, D. V., R. C. MOORE AND F. P. DANN 1941 Effect of pantothenic acid alone and in natural products on nutritional achromotrichia in rats. *Proc. Soc. Exp. Biol. and Med.*, vol. 46, p. 507.
- GYÖRGY, P., AND C. E. POLING 1940 Pantothenic acid and nutritional achromotrichia in rats. *Science*, vol. 92, p. 202.
- GYÖRGY, P., C. E. POLING AND Y. SUBBAROW 1940 Observations on the factor curative of nutritional achromotrichia. *J. Biol. Chem.*, vol. 132, p. 789.
- JKES, T. H. 1940 Reproduction in rats on synthetic B-complex supplement. *Proc. Soc. Exp. Biol. and Med.*, vol. 45, p. 625.
- LUNDE, G., AND H. KRINGSTAD 1939 Über veränderungen des pelzes von ratten durch mangel an gewissen faktoren des vitamin B-komplexes. II. *Z. Physiol. Chem.*, vol. 257, p. 201.
- MORGAN, A. F., B. B. COOK AND H. G. DAVISON 1938 Vitamin B<sub>2</sub> deficiencies as affected by dietary carbohydrate. *J. Nutrition*, vol. 15, p. 27.

- MORGAN, A. F., AND H. D. SIMMS 1940 Greying of the fur and other disturbances in several species due to a vitamin deficiency. *J. Nutrition*, vol. 19, p. 233.
- NIELSEN, E., J. F. OLESON AND C. A. ELVEHJEM 1940 Fractionation of the factor preventing nutritional achromotrichia. *J. Biol. Chem.*, vol. 133, p. 637.
- RICHARDSON, L. R., A. G. HOGAN, B. LONG AND K. I. ITSCHNER 1941 The number of vitamins required by the rat. *Proc. Soc. Exp. Biol. and Med.*, vol. 46, p. 530.
- UNNA, K. 1940 a Pantothenic acid requirement of the rat. *J. Nutrition*, vol. 20, p. 565.
- 1940 b Effect of pantothenic acid on growth and reproduction of rats on synthetic diets. *Am. J. Med. Sci.*, vol. 200, p. 848.
- 1941 The effect of pantothenic acid on achromotrichia in rats. *Am. J. Physiol.*, vol. 133, p. P 473.
- UNNA, K., AND W. L. SAMPSON 1940 Effect of pantothenic acid on the nutritional achromotrichia. *Proc. Soc. Exp. Biol. and Med.*, vol. 45, p. 309.
- WILLIAMS, R. R. 1940 Inefficacy of pantothenic acid against the greying of fur. *Science*, vol. 92, p. 561.



# INEFFICACY OF HORMONES IN NUTRITIONAL ACHROMOTRICHIA OF RATS<sup>1</sup>

CHARLES W. MUSHETT AND KLAUS UNNA

*Department of Biology, New York University, and the  
Merck Institute for Therapeutic Research, Rahway, New Jersey*

ONE FIGURE

(Received for publication July 16, 1941)

Greying of the fur of young black and piebald rats produced by dietary deficiency in pantothenic acid can be prevented by the addition of calcium pantothenate (György and Poling, '40; Unna and Sampson, '40). Also, it has been shown (Unna, Richards and Sampson, '41) that the effectiveness of liver and rice bran depends largely on the amount of pantothenic acid present in such preparations. Morgan and Simms ('40) have reported that the achromotrichia of rats maintained on diets free from "filtrate factor" can be cured by the administration of extracts of the thyroid and of the adrenal cortex. Since pathologic changes have been found in the adrenals of rats maintained on diets deficient in pantothenic acid (Daft and Sebrell, '39; Morgan and Simms, '39; Ashburn, '40), it seemed of interest to study the effect of the administration of hormones to pantothenic acid deficient rats with particular reference to their influence on adrenal hemorrhages and achromotrichia.

Black and piebald rats of a uniform strain were placed at 3 weeks of age on a synthetic diet consisting of dextrose 68%; casein, vitamin free 18%; hydrogenated vegetable oil<sup>2</sup> 8%; salt mixture U.S.P. XI no. 1, 4%; cod liver oil 2%; and supplemented with 0.8 mg. each of thiamine, riboflavin and pyridoxine, 10 mg. of nicotinamide and 100 mg. of choline

<sup>1</sup> Submitted by Charles W. Mushett in partial fulfillment of the requirements for the degree of Master of Science, New York University.

<sup>2</sup> Crisco.

chloride per 100 gm. of diet. In order to facilitate the detection of changes in the hair color, the rats were depilated with barium sulfide at the beginning of the experiment. A thick paste of barium sulfide was rubbed into the fur for about 1 minute, and instantaneous rinsing in warm water removed the hair without causing irritation of the skin. No attempt was made to remove the hair of the head.

In preventive tests, the hormone preparations were administered daily from the beginning of the dietary regime. In each series the number of control and test animals was the same. Weight and symptom records were kept on all animals, and autopsies were performed after death. Curative effects were studied by daily administration of the hormone preparation to rats which had become completely grey after being maintained on the basal diet for at least 4 weeks. In evaluating the efficacy of a hormone, part of a group of grey rats received the hormone, another part an effective dose of calcium pantothenate (100 µg.), and the remainder were left untreated on the basal diet.

Sketches of the distribution of pigment in the skin and of the growth and color of the fur were made twice weekly for every rat on special protocol sheets stamped with a rat's body outline.

The hormone preparations used in this study were: adrenal cortical extract,<sup>3</sup> desoxycorticosterone acetate,<sup>4</sup> thyroid U.S.P.,<sup>5</sup> and anterior pituitary extract.<sup>6</sup> All preparations were found to be free from pantothenic acid by microbioassays on *Lactobacillus casei* (Snell, Strong and Peterson, '37).

Young black rats on the basal diet without depilatory treatment developed distinct grey patterns within 4 weeks and became entirely grey about the fortieth day. The rats which were depilated at the beginning of the dietary regime, gave evidence of greying in an average of 14 days and were wholly

<sup>3</sup>"Cortin" Roche-Organon—1 cc. equivalent to 50 gm. of fresh adrenal cortex.

<sup>4</sup>"Doca", Roche-Organon.

<sup>5</sup>Lilly.

<sup>6</sup>Squibb.

or mostly covered with grey hair within 24 days (fig. 1). No differences in either growth or the appearance of other symptoms characteristic of pantothenic acid deficiency were observed between these rats and those which had not been depilated. After depilation hair started to grow within 2 to 3 days beginning on the ventral body surface and progressing dorsally up the sides where it met at the midline about two-

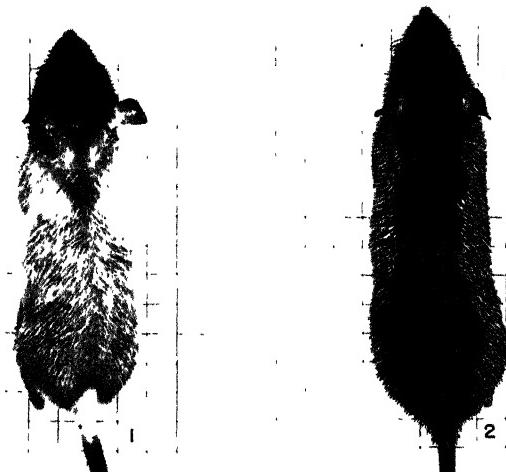


Fig. 1 Greying of the fur after 25 days on a pantothenic acid free diet:  
1—Rat depilated at the beginning of the dietary regime showing uniformly grey fur; 2—Rat not depilated showing development of greying in distinct pattern.

thirds anteriorly. From there it progressed caudally and toward the neck. The last areas to become covered were the nape of the neck and the base of the tail. This sequence of hair growth was common to all groups. It coincides with the growth of hair observed by Butcher and Richards ('39) in well-fed albino rats. The wave of hair growth is preceded by the appearance of pigment in the skin and pigmentation of the skin has been observed regardless of whether the new hair comes in black or grey.<sup>7</sup>

<sup>7</sup> Observations on the skin pigment will be reported in another communication.

*Prophylactic test*

The results obtained with the daily administration of cortical extract, desoxycorticosterone acetate, anterior pituitary extract and thyroid to rats from the beginning of the dietary regime are summarized in table 1. None of the hormones in

TABLE 1

*Effect of various hormones on the occurrence of grey hair and adrenal hemorrhages in young black rats maintained on a vitamin B complex free diet supplemented with 0.8 mg. each of thiamine, riboflavin and pyridoxine, 10 mg. of nicotinamide and 100 mg. of choline chloride per 100 gm. of diet.*

NO OF ANI-MALS	SUBSTANCE	DAILY DOSE	NO. OF RATS SURVIVING FOR MORE THAN 3 WEEKS	NO. OF RATS SHOWING GREY FUR AT 3 WEEKS	ADRENAL HEMORRHAGE
10	Cortical extract	0.25 cc. intramuse.	6	6	+
5	" "	0.5 cc. "	3	3	+
15	Desoxycorticosterone acetate	2.5 mg. intramuse.	11	10	+
10	Anterior pituitary extract	0.25 cc. intramuse.	3	6	+
10	Anterior pituitary extract	0.5 cc. intramuse.	4	7	+
15	Thyroid U.S.P.	1.0 mg. by mouth	10	8	+
10	" "	5.0 mg. " "	3	3	+
15	" "	10.0 mg. " "	3	4	+
48	Controls	—	35	48	+
45	Calcium pantothenate	100 µg. by mouth	45	0	0

the dosage listed in the table produced any increase in weight of the rats. Rats receiving daily 5 mg. and 10 mg. of thyroid reached an average weight of only 45 and 40 gm. respectively, whereas the average weight at which the other groups and the control animals became stationary was 50 to 70 gm. The mortality of the rats receiving the hormones was somewhat higher than that of the control rats. The administration of thyroid produced toxic effects; seven rats of the group of ten rats receiving 5 mg. of thyroid daily died within 3 weeks, while the same dose of thyroid given daily over a period of

4 weeks was apparently well tolerated by rats maintained on a complete diet. No effect on the rate of growth of new hair after the depilation was observed in rats receiving cortical extract, desoxycorticosterone acetate and anterior pituitary extract. Pigmentation of the skin and growth of new hair occurred somewhat more rapidly in rats receiving thyroid, than in those of the control group. None of the hormones prevented the occurrence of adrenal hemorrhages. All animals surviving for at least 3 weeks showed greying of the fur equal to that of the control rats. On the other hand, the daily administration of 100 µg. of calcium pantothenate prevented adrenal hemorrhages and the depigmentation of the fur.

TABLE 2

*Effect of various hormones on rats with grey fur maintained on a vitamin B complex free diet supplemented with 0.8 mg. each of thiamine, riboflavin and pyridoxine, 10 mg. of nicotinamide and 100 mg. of choline chloride per 100 gm. of diet.*

NO. OF ANI-MALS	SUBSTANCE	DAILY DOSE	DAYS ON BASAL DIET PREVIOUS TO TREATMENT	PERIOD OF TREATMENT IN DAYS	EFFECT ON GREY HAIR
3	Cortical extract	0.25 cc. intramuse.	25	43, 9, 9	None
4	" "	0.5 cc. "	25	43, 42, 42, 22	"
4	" "	0.5 cc. "	33	56, 56, 36, 21	"
4	" "	0.5 cc. "	54	36, 32, 23, 14	"
4	Desoxycorticosterone acetate	0.25 mg. "	45	40, 35, 32, 9	"
4	Desoxycorticosterone acetate	1.0 mg. "	45	32, 32, 32, 14	"
7	Anterior pituitary extract	0.5 cc. "	49	52, 41, 41, 40, 15, 13, 9	"
6	Thyroid, U.S.P.	1.0 mg. by mouth	49	43, 37, 34, 37, 23, 20	"
7	" "	5.0 mg. " "	49	27, 21, 16, 13, 9, 9, 9	"
3	" "	10.0 mg. " "	40	20, 20, 13	"
	Calcium pantothenate	100 µg. by mouth			Black pigmentation restored

*Curative test*

The results obtained with the daily administration of hormone preparations to rats which had developed grey hair on the basal diet are summarized in table 2. No effect on achromotrichia has been observed on treating the animals with the various preparations over periods extending to 56 days. Adrenal hemorrhages were found in all groups of rats treated with these substances. None of the preparations were capable of prolonging the life of the grey rats. Calcium pantothenate, on the other hand, produced a marked effect on the achromotrichia within 3 weeks, and within 4 to 6 weeks the pigmentation of the fur was practically restored.

## DISCUSSION

While the basal diets employed by Morgan and Simms were essentially the same as ours, the method of administering the supplements was different in that we fed all of the supplementary vitamins from the beginning of the test, whereas Morgan and Simms ('40) supplemented their diet first with only thiamine until growth ceased and then added their riboflavin and pyridoxine supplements. This might account for the greater survival time of their animals. It required 56 to 70 days for their rats to begin to show the characteristic changes of the fur. With our procedure, the majority of rats die within 5 to 6 weeks and only a few survive for 10 to 13 weeks. Furthermore, it might be possible, that the thyroid and cortical extracts used by these authors might have contained traces of pantothenic acid.

## CONCLUSIONS

The effect of adrenal cortical extract, desoxycorticosterone acetate, thyroid and anterior pituitary extract has been studied on black and piebald rats maintained on a vitamin B complex free diet supplemented with thiamine, riboflavin, nicotinamide, pyridoxine and choline.

1. Daily administration of adrenal cortical extract (0.25 and 0.5 cc.), desoxycorticosterone acetate (2.5 mg.), thyroid

(1, 5 and 10 mg.) and anterior pituitary extract (0.25 and 0.5 cc.) to young rats from the beginning of the dietary regimen failed to prevent the greying of the fur or the occurrence of adrenal hemorrhages and other lesions encountered in rats on diets deficient in pantothenic acid.

2. Daily administration of adrenal cortical extract (0.25 and 0.5 cc.), desoxycorticosterone acetate (0.25 and 1 mg.), thyroid (1, 5 and 10 mg.) and anterior pituitary extract (0.5 cc.) over periods extending to 56 days to rats which had been rendered grey by the dietary regimen, failed to restore the black pigmentation of the fur.

#### LITERATURE CITED

- ASHBURN, L. L. 1940 The effect of administration of pantothenic acid on the histopathology of the filtrate factor deficiency in rats. Public Health Reports, vol. 55, p. 1337.
- BUTCHER, E. O., AND R. A. RICHARDS 1939 The relation of the adrenals to the retarded hair growth in underfed albino rats. Endocrinology, vol. 25, p. 787.
- DAFT, F. S., AND W. H. SEBRELL 1939 Hemorrhagic adrenal necrosis in rats on deficient diets. Public Health Reports, vol. 54, p. 2247.
- GYÖRGY, P., AND C. E. POLING 1940 Pantothenic acid and nutritional achromotrichia in rats. Science, vol. 92, p. 202.
- MORGAN, A. F., AND H. D. SIMMS 1939 Adrenal atrophy and senescence produced by a vitamin deficiency. Science, vol. 89, p. 565.
- 1940 Greying of the fur and other disturbances in several species due to a vitamin deficiency. J. Nutrition, vol. 19, p. 233.
- SNELL, E. E., F. M. STRONG AND W. H. PETERSON 1937 Growth factors for bacteria. VI. Fractionation and properties of an accessory factor for lactic acid bacteria. Biochem. J., vol. 31, p. 1789.
- UNNA, K., AND W. L. SAMPSON 1940 Effect of pantothenic acid on nutritional achromotrichia. Proc. Soc. Exp. Biol. and Med., vol. 45, p. 309.
- UNNA, K., G. RICHARDS AND W. L. SAMPSON 1941 Studies on nutritional achromotrichia in rats. J. Nutrition, vol. 22, p. 553.



## DIGESTIBILITY AND BIOLOGICAL VALUE OF WHOLE WHEAT BREADS AS COMPARED WITH WHITE BREAD

JOHN R. MURLIN, MARGARET E. MARSHALL AND  
CHARLES D. KOCHAKIAN

*Department of Vital Economics, University of Rochester, New York*

ONE FIGURE

(Received for publication July 25, 1941)

The most ancient and honorable of man's foods, bread is of interest at the present time for what it does not contain of the nutrients from wheat rather than for what it does contain. The complete realization of the fact that white flour lacks both minerals and vitamins contained in the wheat berry has led at last to governmental recommendations calling for restoration of the lost substances. Thus far, however, no emphasis has been placed on the relative values of the proteins contained in the entire grain as compared with the endosperm alone. The evidence is not adequate.

Klein, Harrow et al. ('26) made a study on rats of the nutritive value of the various layers of both wheat and corn. It was already known that the various layers of the cereal grains differed not only in their vitamin content and in their content of inorganic salts, but also in their general protein make-up. These authors found that the fractions which contain the pericarp and the germ, and which serve as the distinguishing ingredients of the dietetically superior whole grain flour, possess a much higher nutritive value for rats than fractions which are composed mainly of the endosperm. The milling fraction known as "red dog" gave the highest biological value of any of the fractions studied.

Boas-Fixsen and Jackson ('32) found that proteins of ground whole wheat of the soft Red English variety had by the nitrogen balance method a biological value of 68 in rats, when fed at the level of 6% protein. Wheat germ and endosperm of the same wheat fed at 7% level gave B.V.'s of 69 and 61. In 1934 the same workers with J. C. D. Hutchinson studied the protein values of the same whole wheat and whole yellow maize (from South America) fed at near 10% level, by the rat growth method, and found the rate of growth for the former to be 1.36 gm. per gram of protein fed, and for the latter 1.29 gm. When suitable deduction was made for the protein required for maintenance the values for growth alone were 1.85 and 1.73. Wheat apparently is slightly better than maize as a source of protein both for growth and maintenance of the rat. That the level of protein is very important is shown by the further work of Chick, Boas-Fixsen, Hutchinson and Jackson ('35). Whole wheat at 3.6% level gave 100% B.V., and at 5.6% level only 68; wheat germ at 3.6% level gave 90%, and at 6.8% level 69.

There is only one study available on biological values of breads in the modern sense, namely, the very excellent short paper of French and Mattill ('35). In this study the biological values for the proteins of white bread, "whole wheat" (50-50, meaning 50% of white flour and 50% whole wheat) and of rye bread (25-75) were determined both on rats by Mitchell's method and on human subjects in comparison with whole milk as the standard protein. On mature rats white bread protein had a biological value of 83, "whole wheat" 80, and rye bread 82. The average milk replacement value for six human subjects on the same breads was exactly the same for all the breads, namely, 83. Milk itself, however, has a biological value of about 83 to 86 (Boas-Fixsen, '34-'35).

It seemed worth while, therefore, to make a new study of the biological value of some truly whole wheat breads and of white breads by using whole egg as a standard protein, a method previously used in this laboratory (Murlin, Nasset and Marsh, '38) for the study of cereal breakfast foods on human subjects. Whole egg proteins fed at a 5% level of

intake have given the highest biological value<sup>1</sup> of any known human food when fed to adult rats (Mitchell and Carman, '24; Sumner, '38). The occasion for such a study came with the invention of a new method of removing the rough outer part of the bran from wheat—the flotation process of Earle.<sup>2</sup>

TABLE 1  
*The diet squad and calorie distribution of diet.*

GROUP a <sup>1</sup>	AGE	HEIGHT	SURF. AREA	BEG. WEIGHT	WEIGHT CHANGE	Total intake	CALORIES		
							P.	CHO	F.
		cm.	sq. M.	kg.	kg.		%	%	%
1. R.R.	27	167.5	1.78 <sup>1</sup>	68.8 <sup>1</sup>	-0.8	2969	4.8	49.2	46.0
2. T.H.L.	34	162.5	1.57 <sup>2</sup>	54.5 <sup>2</sup>	-3.4	2453	4.6	48.0	47.4
3. D.F.	25	182.9	1.85 <sup>3</sup>	65.2 <sup>3</sup>	-2.0	2731	4.9	47.9	47.2
4. J.P.L.	26	182.3	2.03 <sup>4</sup>	81.3 <sup>4</sup>	-2.3	3822	4.4	48.0	47.6
5. D.H.B.	29	177.8	1.96 <sup>5</sup>	76.9 <sup>5</sup>	-0.1	2982	5.3	48.0	46.7
GROUP b <sup>1</sup>									
6. C.D.K.	32	170.2	1.78 <sup>1</sup>	67.5 <sup>1</sup>	+0.9	2834	5.0	48.0	47.0
7. E.S.N.	41	171.5	1.90 <sup>6</sup>	77.6 <sup>6</sup>	-0.3	3021	5.3	47.9	46.8
8. R.A.B.	24	190.0	2.04 <sup>4</sup>	79.9 <sup>4</sup>	+0.8	3561	4.6	50.4	45.0
10. F.L.T.	25	163.0	1.62 <sup>2</sup>	58.0 <sup>2</sup>	0.0	2415	4.9	50.5	44.6
11. L.E.E.	26	177.8	1.85 <sup>3</sup>	68.5 <sup>3</sup>	-0.2	3151	4.5	48.0	47.5
Average	29	176.7	1.84	69.8	-0.8	2914	4.8	48.6	46.6

<sup>1</sup> Note that subjects in the two groups *a* and *b* may be paired well, as indicated by small index figures, for weights and surface area.

For the determination of biological value of the proteins and their over-all digestibility human subjects were chosen because of the direct applicability of the results to current

<sup>1</sup> Biological values for whole egg proteins have not been determined for adult men by the most modern methods. That it is decidedly superior to milk was shown by Sumner, Pierce and Murlin ('38).

<sup>2</sup> U. S. patent No. 2,143,306. This process removes only the thin epidermis of the wheat berry, leaving intact in the "peeled wheat" all nutrients of the wheat grain available to man. At the invitation of the Continental Baking Company, which controls the Earle patents on this process, the experiment to be described was begun on January 14th and was concluded on March 27th, 1941. The entire study contemplated a complete assay of the B-complex vitamins, and studies also on the relative rates of digestion of the different kinds of bread, which will be reported later. The invitation was accepted in the belief that the information to be gained would be of timely importance nationally.

national problems in nutrition. The squad consisted at the start of eleven men, all apparently in good health, but one was obliged to withdraw at the end of the eighth period because he felt that the diet did not sufficiently maintain the vigor of body and mind necessary to keep up his work. All were members of the institution as graduate or medical students or as members of the departmental staff. Table 1 describes their physical characteristics, their individual weight changes during the experiment, the total calorie intake, and the distribution of calories chosen, within a narrow freedom of choice, by each person.

The diet was planned to supply, as nearly as possible, 80% of the food nitrogen from whole egg, as standard protein, and 80% from bread in alternate periods of 6 days each. Approximately 10% was contained in butter and cream,<sup>3</sup> and 10% in the fruits, vegetables, and accessories as indicated in the sample diet shown in table 2. Also illustrated are the amounts of each constituent, the nitrogen supplied by each, the grams carbohydrate, and the total calories. The choice of a rather high fat diet was dictated by the necessity of supplying sufficient energy without excessive bulk or too much sweetness. The protein level was kept at, or very near, 5% of the total energy, on the well known principle that the body makes more economical use of protein the lower the supply. No subject varied at any time from his average of protein more than a few tenths of 1%, or from his average for carbohydrate and fat more than 2% or 3%. There were a few minor changes

<sup>3</sup> The 4x cream was obtained by direct daily delivery from the same herd of cows on the Markham-Puffer farm near Avon, New York. Eggs were obtained also by frequent delivery from the same flock of hens fed throughout on the same feed. The butter was obtained from the Beatrice Creamery Company, and kept in the Hospital refrigerated storage room. The applesauce was obtained from the W. N. Clark Canning Company, and represented a fixed mixture of New York-State-grown Twenty Ounce, Greenings, and Baldwins. Orange juice was expressed daily from oranges, one-half of which were purchased locally and one-half donated through the local representative of the California Fruit Growers' Association. Lettuce was the iceberg variety of varied import. Coffee was Chase and Sanborn brand. It will be observed that no account is taken of the coffee nitrogen as intake because in the experience of this laboratory it is all secreted by the kidneys of healthy men. It was, therefore, deducted from the daily nitrogen output in the urine.

in total calories to keep weight changes low, but adjustments were always made as to percentage distribution.

The breads chosen for comparison were: (1) a well known breakfast cereal<sup>4</sup> which, while not a bread in the strictest sense, is, nevertheless, a truly whole wheat "baked" product; (2) a local "100% whole wheat bread" which, it was later found, contained 5% of non-fat milk solids from buttermilk

TABLE 2  
Sample diet (C.D.K., net weight 67.5 kg.).

	EGG—PERIOD III			LEAN WHITE BREAD—PERIOD IV				
	Gm.	N	CHO	Cal.	Gm.	N	CHO	Cal.
Egg <sup>1</sup>	222	4.51		333.1	Bread,			
4x cream	200	.551	5.8	722.9	lean white	314	4.68	169.1 820
Butter	24	.024		188.8	Butter	62	.062	476.8
French dressing	9	.005	.3	61.7	4x cream	183	.586	5.3 659.9
Lettuce	60	.08	1.8	10.8	Lettuce	120	.16	3.6 21.6
Applesauce	200	.04	48	196	French			
Orange juice	296	.326	38.6	166	dressing	30	.015	.8 205.5
Karo	26	.005	20	80	Applesauce	300	.06	72 294
Sugar	75		75	300	Orange juice	318	.35	41.4 165
Corn starch					Karo	33	.01	25 100
biscuit <sup>2</sup>	3X <sup>3</sup>	.09	151	774	Sugar	23		23 92
Coffee	3X				Coffee	3X		
			5.63	340.5 2833.3				5.92 340.2 2834.8
Protein		5%			Protein			4.86%
Fat		47.0			Fat			47.1
Carbohydrate		48.1			Carbohydrate			48.0

<sup>1</sup>Eaten principally as whole egg plain omelet.

<sup>2</sup>Contained a small amount of the egg allowance.

<sup>3</sup>Times daily.

based upon the flour weight; (3) the whole wheat bread<sup>5</sup> baked<sup>5</sup> from the "peeled wheat" flour (see footnote 2, p. 575); (4 and 7) two lean white breads differing decidedly in their analyses purchased from local chain stores; (5) the whole wheat bread baked<sup>5</sup> from "peeled wheat" with high vitamin yeast,<sup>6</sup> and (6) a white bread containing 5% non-fat milk solids based upon the flour; and made with high vitamin yeast.<sup>6</sup>

<sup>4</sup>Shredded Wheat Biscuit.

<sup>5</sup>By the Continental Baking Company.

<sup>6</sup>High vitamin yeast, made by the Fleischmann Laboratories, contained the following per pound of the baker's yeast in the moist state: Iron 9.1 mg., nicotinic acid 82 to 103 mg., thiamine approximately 90 mg., riboflavin 11.2 mg. It was used in the amount of 3.5 pounds per 100 pounds of the flours.

## RESULTS

The data necessary from each subject and their progressive use in calculation of true digestibility and biological value of the proteins in one of the breads are illustrated in table 3. Urines were analyzed only for the last 4 days of each period. Feces were separated for entire 6-day periods by means of charcoal markers taken at the first meal of each, and by means of the characteristic colors and textures produced by the test foods. All foods containing nitrogen were sampled each day, the samples pooled, and aliquots analyzed by periods.

Bread fecal nitrogen minus the average egg fecal nitrogen for each subject gives the fecal food nitrogen (or, otherwise, excess "alimentary nitrogen") for that bread, on the assumption that no egg nitrogen as such reaches the feces. This assumption is not completely borne out by the facts, for occasionally traces of characteristically colored whole egg were seen in the feces, but it is justified by the consistency of the figures for the egg fecal nitrogen for each subject in all the seven periods and by the fact that these figures agree very closely with those in white bread feces, which notoriously contains minimal food nitrogen.

Subtracting fecal food nitrogen from the total nitrogen intake of the bread diet for each subject gives the absorbed nitrogen, and the percentage which this represents of the total is the true digestibility of the protein being studied, other constituents of the diet remaining constant. The slight variations from egg to bread period in the low-nitrogen constituents, made necessary to keep percentages of protein, fat, and carbohydrate equal, are illustrated in table 2.

Biological value of the bread proteins is determined by the excess of the urine nitrogen of the bread period over that of the egg periods, expressed as a percentage of the absorbed nitrogen and subtracted from 100. Table 3 shows that the average B.V. of the local "100% whole wheat bread" which contained 5% non-fat milk solids is 77.8 with a mean deviation for the ten subjects of  $\pm 5.5$ , and standard deviation of 6.51.

TABLE 3  
*Biological value of local "100% whole wheat bread" + 5% non-fat milk solids  
 (based on daily averages).*

SUBJECT	BREAD FECAL N	AV. EGG FECAL N	a - b FECAL FOOD N	c	d	e	f	g	h	i	j	k	TRUE DIGESTIBILITY ABS. N X 100 TOTAL FOOD N	AV. BREAD URINE N	AV. EGG URINE N	g - h EXCESS URINE N	EXCESS N X 100 ABSORBED N	B.V. 100 - j	DEV. FROM MEAN
													gm.	gm.	gm.	gm.	%	%	
Period I	1	1.70	0.99	0.71	5.79	5.08	87.7	5.85	4.46	1.39	27.4	72.6	-5.2						
	2	1.19	0.83	0.36	4.58	4.22	92.1	4.29	3.35	.94	22.3	77.7	-0.1						
	3	1.45	1.10	0.35	5.47	5.12	93.6	6.20	4.53	1.67	32.6	67.4	-10.4						
	4	1.86	1.23	0.63	6.89	6.26	90.8	7.43	5.48	1.95	31.1	68.9	-8.9						
	5	1.28	1.08	0.20	6.41	6.21	96.9	6.21	4.65	1.56	25.1	74.9	-2.9						
Period II	6	1.41	1.02	0.39	5.98	5.59	93.5	5.29	4.11	1.18	21.1	78.9	+1.1						
	7	1.30	1.04	0.26	6.88	6.62	96.2	6.14	4.96	1.18	17.8	82.2	+4.4						
	8	1.49	1.00	0.49	7.06	6.57	93.0	6.23	5.42	0.81	12.3	87.7	+9.9						
	9	1.19	0.71	0.48	5.10	4.62	90.6	4.49	3.68	0.81	17.5	82.5	+4.7						
	10	1.38	1.00	0.38	6.08	5.70	93.7	5.22	4.40	0.82	14.4	85.6	+7.8						
Period III	11	1.38	1.00	0.42	6.02	5.60	92.8	5.73	4.50	1.23	22.2	77.8	Av. ± 5.5						
	12	1.42	1.00	0.42	6.02	5.60	92.8	5.73	4.50	1.23	22.2	77.8	Av. ± 5.5	St. D. = 6.51					

It was necessary in this study to use the average fecal and urine nitrogens for all the egg periods instead of for the two egg periods adjacent to each bread period, for the reason illustrated in figure 1. The average egg urine nitrogen for the entire squad declined steadily from the first period to the last. There was no compensating drift in the tendency of

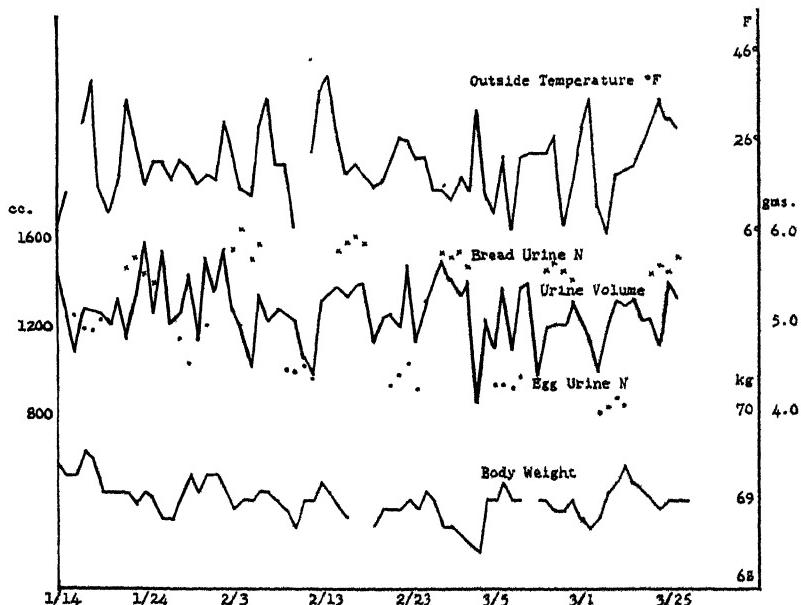


Fig. 1. Graphic comparison of average urine volumes with outside temperature during the course of the experiment. There is no close correlation. The average body weight is shown to increase in egg periods and to decrease in bread periods. There is a progressive decrease in average egg urine nitrogen (dots) with no compensatory change in bread periods (crosses).

the bread proteins. Hence, to compare a bread at the beginning of the series with its associated egg periods would have given the first bread a considerable advantage over the last. This phenomenon of a steady increase in retention of egg protein was not seen in the study of cereal proteins reported in 1938. That study was carried out in late spring and early summer, while this one covered the coldest part of the winter.

No close correlation, however, is to be made out (fig. 1) between outside temperature and either the urinary nitrogen or the urine volume. We are at a loss to explain the difference in the two experiments unless the gradually accumulating deficit of high-value protein caused by a longer and longer dependence on cereal protein for maintenance was greater in the present instance because whole grain products were used in a smaller percentage of the periods.<sup>7</sup>

Average essential data and the computed digestibilities and biological values for all seven of the breads are given in table 4. Only four of the breads, however, namely three whole-wheat and one lean white loaf were eaten by all ten of the subjects. Only these four, therefore, are strictly comparable. The comparative digestibility is indicated by the fecal food nitrogen in column c. The roughest of the whole wheat products, the whole wheat cereal biscuit, gives the highest fecal food nitrogen, and therefore the lowest absorption. Since the average food N was substantially the same for all the breads, this produces also the lowest true digestibility.

The two whole wheat true breads differed in two respects. The local bread contained all of the bran and also 5% non-fat milk solids on the basis of dry flour and added as buttermilk. The new bread lacked the epidermis of the wheat, constituting about 2% by weight of the grain, but no milk solids. It is a little surprising, therefore, that the average fecal food nitrogen is exactly the same for the two, and the consequent true digestibility the same. Apparently the removal of the fibrous outer epidermis in the one has compensated for the digestibility advantage of the added 5% milk solids in the other. As would be expected, the lean white loaf produced the lowest fecal food nitrogen and the highest digestibility.

Some of our foremost nutritional authorities have fallen into the fallacy that highest digestibility of protein confers

<sup>7</sup> The percentage levels of the egg protein calories were nearly identical in the two studies. There were, however, in the former studies some larger additions to total calorie intake in later periods which may have influenced the N balances slightly.

TABLE 4  
*Average digestibilities and biological values of seven breads  
 (based on daily averages).*

NO AND NAME OF BREAD	NO. OF SUB- JECTS	BREAD YECAL N	FECAL FOOD N	TOTAL FOOD N	gm.	(d-c) ABS'D N	e x 100 d TRUE DIGESTIBILITY	BREAD URINE N	EXCESS N	$\frac{e \times 100}{6}$	R.V. 100-i	SP. DEV.	REMARKS
1. Whole wheat cereal biscuit <sup>1</sup>	10	1.89	0.89	6.00	5.12	85.2	5.46	0.96	18.4	81.6	8.5		
2. Whole wheat bread, 5% non- fat milk solids	10	1.42	0.42	6.02	5.60	92.8	5.73	1.93	22.2	77.8	6.5		
3. Peeled wheat bread	10	1.42	0.42	6.06	5.64	93.0	5.65	1.15	20.4	79.6	4.1	Extra B vitamins, see text, p. 584.	
4. Lean white bread I	10	1.03	0.03	6.09	6.06	99.4	6.00	1.50	24.7	75.3	4.4	Same extra B vitamins see text, p. 584.	
3. Peeled wheat (ordinary yeast)	5 (a)	1.34	0.30	6.03	5.73	94.0	5.77	1.98	22.1	77.9	5.8	No extra B vitamins	
5. Peeled wheat (high vit. yeast)	5 (b)	1.27	0.32	6.11	5.79	93.6	5.62	1.11	19.0	81.0	5.6	See footnote 6, p. 577	
6. White bread (with high vit. yeast and non- fat milk solids)	5 (a)	1.11	0.06	5.84	5.78	98.9	5.68	1.19	20.2	79.8	3.7	See footnote 6, p. 577	
7. Lean white bread II	5 (b)	1.07	0.11	6.12	6.03	98.3	6.04	1.53	25.3	74.6	3.9	No extra B vitamins	

<sup>1</sup> Shredded Wheat of National Biscuit Company.

greatest nutritive value. It is evident from the table that of the four breads now being considered the one with lowest digestibility produced the highest B.V. It must be remembered that the same average egg urine nitrogen (column h, table 3) was subtracted from the several bread urine nitrogens in table 3 to get excess nitrogen. Thus comparing the two whole wheat breads eaten by ten subjects, we find that one gave a slightly higher absorbed N but produced slightly less urine nitrogen, and therefore showed a higher B.V. The difference is not statistically significant, but it illustrates the fact that a lesser absorption may be succeeded by a greater retention which in reality gauges B.V. The fact that one of these whole wheat breads contained the added milk protein might explain why the B.V. of the other, lacking the epidermis of the grain, was not significantly greater.

The second whole wheat bread (no. 3 of table 4) in comparison with the lean white loaf I shows 0.42 gm. less absorption but shows only 0.35 gm. less urine N, and yet the percentage of the absorbed N which the excess N represents is, for the white bread, over 4.3% greater than for the whole wheat—a difference which, reflected in the B.V., is statistically significant. The difference between the B.V.'s of the whole wheat biscuit and lean white bread also is statistically significant, but not the difference between those of the local whole wheat bread and the lean white even though the former carried 5% milk solids.

The last four comparisons in table 4 were made on only five subjects for each bread. The subgroup *a* contained the first five subjects and subgroup *b* the second five listed in tables 1 and 3. The object of these periods was to get some light on the influence of "high vitamin yeast"<sup>8</sup> compared with ordinary yeast in the baking of a whole wheat bread and of white bread. The first comparison indicates that the high vitamin yeast in the peeled wheat bread conferred a higher B.V. than the same bread with ordinary yeast. We have no evidence that one of the subgroups regularly produced a

<sup>8</sup> See footnote 6, page 577.

higher B.V. than the other. Furthermore, in comparing different sources of the B-complex (see below) the source<sup>9</sup> which proved superior with one group did so with the other.

The white bread containing the high vitamin yeast also gave a higher biological value than the lean white loaf containing ordinary yeast, and the difference is great enough to be statistically significant for a larger number of subjects. The white bread baked with high vitamin yeast, however, contained also 5% non-fat milk solids, and this constituent may have added to its biological value. It is well known (Mitchell and Carman, '26) that milk and white flour proteins do supplement each other when fed together in the proportion of 1 to 2. The proportion of milk to flour proteins in the bread is much lower than this and therefore the exact effect of its milk protein can only be conjectured. More work should be done on these supplementary effects.

*Effect of extra vitamins on biological value.* An attempt was made to learn whether extra B vitamins added to a diet already approximately adequate in these factors would improve the B.V. of the cereal proteins. Accordingly in the third bread period when one of the lean white breads and the peeled wheat bread were eaten by groups *a* and *b* respectively, a quantity of a certain concentrate<sup>10</sup> sufficient to supply 750 I.U. of thiamine, 0.9 mg. of riboflavin, 300 µg. of pyridoxine, 30 rat growth units of filtrate factors and nicotinic acid equivalent to 37 gm. fresh whole liver was given to each man of both subgroups for 4 of the 6 days. For the last 2 days the vitamin concentrate was a different one,<sup>9</sup> the daily dose containing approximately 500 I.U. of thiamine, 125 Sherman units of riboflavin and other members of the B-complex from yeast.

In the following egg period and the fourth bread period when the same breads now were eaten by groups *b* and *a* respectively (the reverse of bread period 3) extra vitamins were continued in approximately one-half the amount (i.e.,

<sup>9</sup> Harris' "Yeast Vitamine", 7½ grain tablets.

<sup>10</sup> Lederle's "Vitamin B complex, oral".

250 I.U. of B, and 62 Sherman units of B<sub>2</sub>). The two groups in any period were receiving the same extra vitamins. It is of interest that in these two periods combined the B.V. of the peeled wheat bread was higher than when the same bread (second peeled wheat bread 3 in table 4) was eaten without extra vitamins. Also the B.V. of the lean white bread 4 eaten with extra vitamins was higher than that of lean white bread 7 eaten without extra vitamins. The difference in neither case is statistically significant, but probably would prove to be so with more subjects.

*Economic considerations.* In 1918 a report was issued by a committee of the Royal Society of London entitled "The Digestibility of Breads", from which it was concluded on the basis of the yearly consumption of wheat in the United Kingdom in the years immediately preceding the first world war, that if all of the population had eaten the bread made from 90% extraction flour instead of the bread made from 80% extraction flour there would have been a yearly saving of calories equal to an extra month's supply for the whole United Kingdom.

The saving from the use of the peeled wheat bread used in this study as compared with the lean white bread I is indicated in table 5.

From the comparison shown in this table it is evident that the saving would amount to 93,737 kg.-calories, or enough to support the average person (2200 cal.) for over 40 days.

But this is not the entire advantage of whole wheat. If the average person in the United States who now eats lean white bread were to change to an acceptable whole wheat bread such as the peeled wheat bread which was the nearest to 100% of any true bread used in this investigation, there would result not only this large saving in calories per person in the country's human food supply, but in addition the consumer would get the entire B-complex of vitamins contained in wheat, more minerals, especially calcium, phosphorus and iron, and his tissues would have the advantage of a biological

value of the protein significantly higher than that of the lean white bread.

These are considerations too important to be overlooked. The country is awake to the importance of better nutrition; it should be awake also to the importance of vital economy in nutrition.

TABLE 5  
*Computation on calorie basis of saving from use of whole wheat.<sup>1</sup>*

PEELED WHEAT BREAD	LEAN WHITE BREAD
Composition as eaten.	
N = 1.69%, Protein 9.633%	1.43% Protein 8.125%
Carbohydrate (by hydrolysis)	38.4%
Fat (petroleum ether extract)	3.21%
Digestibility.	
Protein	93.0%
Carbohydrate	98.6%
Fat	97.1%
Computation of calories.	
Protein $9.633 \times .93 \times 4.1 = 36.73$	$8.151 \times .994 \times 4.1 = 33.22$
Carbohydrate $38.4 \times .986 \times 4.1 = 155.22$	$48.0 \times .997 \times 4.1 = 196.18$
Fat $3.21 \times .971 \times 9.3 = 28.99$	$2.6 \times .974 \times 9.3 = 23.55$
Total per 100 gm.	220.94
Calories per pound	1003.1
100 pounds wheat produce 98 pounds flour	69.4 pounds flour
98 pounds flour produce 158 pounds bread	104 pounds bread
$158 \times 1003.1 = 158.490$ calories	119.433 calories
Taking 240 pounds (4 bushels) as average per capita consumption, <sup>2</sup>	
$2.4 (158.490 - 119.433) = 93.737$ calories saved.	

<sup>1</sup> The two breads chosen are the ones used in this investigation which were eaten by the entire squad and showed the greatest contrast in nutrient qualities.

<sup>2</sup> A recent bulletin (mimeographed) from the U. S. Department of Agriculture entitled "Consumption of Agricultural Products" gives the average per capita consumption of flour, for the 10 years 1930-1939, as pounds of wheat, at 226.

#### SUMMARY AND CONCLUSIONS

Digestibilities and biological values of the proteins of whole wheat and white breads were determined on ten healthy male subjects averaging 29 years of age.

Average daily intake of energy was 2914 kg. cal., distributed, with slight variations, 4.8% to protein, 48.6% to carbohydrate and 46.6% to fat.

Six-day periods on egg as the source of 80% of the N alternated with like periods on bread as the source of this fraction. Ten per cent of the N in all periods came from cream and butter and 10% from fruits, vegetables, and accessories. Twenty-four-hour urines were analysed on the last 4 days, feces in one lot for the full period.

Three whole wheat breads and one whole wheat cereal biscuit were compared with three white breads. In general the whole wheat breads gave lower true digestibility values for the protein than the white, but at the same time produced higher biological values. One white bread containing 5% non-fat milk solids produced a higher B.V. than a whole wheat with the same milk content. Taking the B.V. of egg proteins as 100% the B.V.'s for the proteins of the four whole wheat breads were: whole wheat cereal biscuit, 81.6; a locally made whole wheat bread containing 5% non-fat milk solids, 77.8; "peeled wheat" bread eaten with extra B vitamins, 79.6; same baked with high vitamin yeast, no other extra vitamins, 81.0; same with ordinary yeast, no extra B vitamins, 77.9. For the white breads: lean white I eaten with the same extra B vitamins as with the "peeled wheat" bread, 75.3; similar bread II eaten with no extra B vitamins, 74.6; white bread containing 5% non-fat milk solids and baked with high vitamin yeast, eaten with no extra B vitamins, 79.8.

The results suggest that making bread with "high vitamin" yeast is of some importance from the standpoint of the biological value of proteins; also that eating extra B-complex vitamins improves biological values.

The authors are grateful to Dr. E. S. Nasset and Mrs. Robert Ryer, III, for assistance at important points in this investigation.

#### LITERATURE CITED

- BOAS-FIXSEN, M. A., AND H. M. JACKSON 1932 The biological value of proteins. IV. The biological values of the proteins of wheat, maize, and milk. Biochem. J., vol. 26, p. 1923.
- BOAS-FIXSEN, M. A., J. C. D. HUTCHINSON AND H. M. JACKSON 1934 V. The comparative biological values of whole wheat, whole maize, and maize gluten measured by the growth of young rats. Biochem. J., vol. 28, p. 592.

- BOAS-FIXSEN, M. A. 1934-1935 The biological value of protein in nutrition. Nutr. Abs. and Rev., vol. 4, p. 447.
- CHICK, H., M. A. BOAS-FIXSEN, J. C. D. HUTCHINSON AND H. M. JACKSON 1935 V. The influence of variations in the level of protein in the diet and of heating the protein on its biological value. Biochem. J., vol. 29, p. 1712.
- FRENCH, R. B., AND H. A. MATTILL 1935 The biological value of the proteins of white, wheat and rye breads. Cereal Chem., vol. 12, p. 365.
- KLEIN, A., B. HARROW, L. PINE AND C. FUNK 1926 The nutritive value of various layers of the wheat and corn kernel. Am. J. Physiol., vol. 86, p. 237.
- MITCHELL, H. H., AND G. G. CARMAN 1924 The biological value for maintenance and growth of the proteins of whole wheat, eggs, and pork. J. Biol. Chem., vol. 60, p. 613.
- 1926 The biological value of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem., vol. 68, p. 183.
- MURLIN, J. R., E. S. NASSET AND M. ELIZABETH MARSH 1938 The egg replacement value of the proteins of cereal breakfast foods, with a consideration of heat injury. J. Nutrition, vol. 16, p. 249.
- REPORT OF THE FOOD (WAR) COMMITTEE OF THE ROYAL SOCIETY ON THE DIGESTIBILITY OF BREADS 1918.
- SUMNER, EMMA E. 1938 The biological value of milk and egg protein in young and mature rats. J. Nutrition, vol. 16, p. 129.
- SUMNER, E. E., H. B. PIERCE AND J. R. MURLIN 1938 The egg replacement value of several proteins in human nutrition. J. Nutrition, vol. 16, p. 37.

# APPARENT DIGESTIBILITY OF CARBOHYDRATES, FATS, AND "INDIGESTIBLE RESIDUE" IN WHOLE WHEAT AND WHITE BREADS

ROBERT R. SEALOCK, DANIEL H. BASINSKI AND JOHN R. MURLIN

*Department of Vital Economics, University of Rochester, New York*

ONE FIGURE

(Received for publication July 25, 1941)

An investigation of the digestibility and biological value of the proteins of white, whole wheat, and peeled wheat breads has been reported in the preceding paper (Murlin, Marshall and Kochakian, '41). It is important that the comparative availability of other nutrients in these breads be established. This is particularly pertinent in view of the present discussion relative to enrichment of flours and breads with some of the B vitamins, and the alternative program of the use of higher and higher extraction flours, and/or higher vitamin yeasts.

Accordingly, we have studied from the material available in the preceding investigation the apparent digestibility of carbohydrate, fat, and the "indigestible residue." Since one of the chief differences between white and whole wheat breads is the higher content of roughage, or "indigestible residue" of the latter, particular attention has been paid to this component.

## EXPERIMENTAL

For the plan of the experiment, the composition of the diet and other aspects, the reader is referred to the preceding paper.

Except in those instances noted in the summary table the various food items were analyzed after drying and grinding.

Sufficient numbers of samples were taken throughout the duration of the experiment so that the final value represents a reasonable average for the entire experiment. Analysis of the feces was made from alcohol-dried aliquots that were thoroughly pulverized for sampling.

Ash was determined by brief combustion at 600°C. in the muffle furnace. In order to determine total carbohydrate, both food and feces samples were refluxed 2 hours in 2% hydrochloric acid. The neutralized hydrolysates were precipitated with zinc hydroxide according to the method of Somogyi ('30). Aliquots were then analyzed by the copper-iodometric method of Shaffer and Somogyi ('33), the final calculations being made from a glucose calibration curve. Admittedly glucose calibration is inadequate for a diet containing mixed carbohydrates; however, for purpose of the comparison desired in this experiment this procedure has proved satisfactory. The fat of the foods and feces was determined as the total petroleum ether soluble material by the wet extraction method of Saxon ('14). In order to insure complete extraction of free fatty acids, hydrochloric acid was added as directed in the original method. Additional aliquots were analyzed for indigestible residues by the fractional method of Williams and Olmsted ('35), the final value being obtained by the addition of the lignin, hemicellulose and cellulose found.

The authors are quite aware of the theoretical limitations attendant upon the use of each of these procedures in given instances. However, in view of the fact that final choice was made on the basis of convenience of the procedure, the particular information desired and other circumstances relating to the conduct of the experiment as a whole, the results should prove entirely adequate.

#### RESULTS

The items used in the diet experiment are listed in table 1 with the composition found by the methods described. The list of breads is identical with the list given in table 4 of the previous paper. The higher ash content and lower carbo-

TABLE 1

*Composition of food items. Per cent of dry weight except where indicated.*

FOOD	DRY WEIGHT	ASH	CARBO- HYDRATE	FAT	PRO- TEIN <sup>1</sup>	INDIGESTIBLE RESIDUE			
						Lignin	Hemi- cellulose	Cellu- lose	Total
%									
Whole wheat cereal biscuit <sup>2</sup>	93.80	1.89	70.20	1.46	11.30	0.900	4.200	4.290	9.39
Locally made whole wheat bread, 5% non-fat milk solids	61.60	3.09	64.40	2.89	15.70	0.887	3.540	2.740	7.17
Peeled wheat bread	62.60	3.57	61.10	5.12	15.50	0.930	3.850	2.990	7.77
Lean white bread I	68.90	2.71	69.70	3.79	11.70	0.083	0.908	1.510	2.50
Peeled wheat bread with high vitamin yeast	67.90	3.27	52.30	5.14	14.40	0.815	4.850	2.610	8.28
White bread, 5% non-fat milk solids, high vitamin yeast	68.60	2.80	61.80	4.57	12.00	0.045	0.727	1.980	2.75
Lean white bread II	67.60	2.64	72.00	3.33	12.50	0.115	1.530	0.950	2.60
Eggs	26.85	3.45	1.95	43.20	48.30				
Applesauce	21.68	0.69	33.50		0.56	0.780	2.060	1.820	4.66
Butter <sup>3</sup>		1.72	1.10	78.27	0.61				
Cream, 4X <sup>3</sup>		0.41	1.83	40.26	1.91				
Corn syrup	75.50	0.92	88.70		0.17				
Lettuce	4.15	12.33	19.40	1.80	7.56	3.970	7.250	8.800	20.02
Orange juice	10.40	4.42	61.90		0.81	0.212	0.972	0.625	1.82
French dressing <sup>2</sup>			25.00	0.31					
Cornstarch biscuit	3.55	86.40	10.10	0.52					

<sup>1</sup> N × 5.7 for cereal products; N × 6.25 for all others.<sup>2</sup> Shredded Wheat Biscuit of National Biscuit Company.<sup>3</sup> Values recorded on fresh basis.

hydrate of the whole wheat breads as compared with the lean white breads are, of course, according to expectation. As ordinarily given in tables of composition the carbohydrate is obtained by difference after adding together the ash, protein, fat, and fiber, and subtracting these from the total dry weight. The values obtained by hydrolysis as noted above are naturally considerably lower for the carbohydrate than those found in most food tables.

The protein content of the cereal products was obtained from the nitrogen by use of the factor 5.7, as is the more recent custom among cereal chemists. All other protein values in table 1 were obtained by using the more familiar factor, 6.25. It is of special interest that all of the whole wheat breads show higher protein content than the white breads, with the exception of the whole wheat cereal biscuit,<sup>1</sup> which is included in this study, as in the previous one, not because it is a bread in the strict sense of the word, but because it represents a truly whole wheat "baked" product. The highest value for protein in this table is the locally made whole wheat bread, which contained 5% non-fat milk solids on the basis of dry flour.

Of particular interest at this time are the values for "indigestible residues" by the fractional method. As pointed out by Williams and Olmsted, the values by this method are considerably higher for all foods containing fiber than those obtained by the old Weende procedure. This difference is quite apparent, for example, in the case of the peeled wheat bread and the locally made whole wheat bread. The former on the dry basis by the fractional method gives a total indigestible residue of 7.7%, whereas with the older procedure the value reported is 1.7% crude fiber. The locally made whole wheat bread shows respective values of 7.17% and 2.04%.

In table 2 is reported the average composition of the feces for the different periods. Since the diet was so designed that the supplementary food items remained comparatively constant throughout, the differences recorded are directly resultant from the test food of a particular period.

By deducting the figures of table 2 from the total intake the per cent utilization (in the older sense), or apparent digestibility (in the newer) has been calculated as shown in table 3. It is of interest that although there is considerable variation in the per cent of mineral absorbed (lean white II only 62.1%, others varied from 70.5 to 84.4%), with the exception of the egg and whole wheat cereal biscuit periods

<sup>1</sup> Shredded Wheat Biscuit of the National Biscuit Company.

TABLE 2  
*Analysis of feces. Average values for 6 day periods.*

EXPERIMENTAL FOOD	NO. OF SUBJECTS	TOTAL WEIGHT	SOLIDS %	ASH gm.	CARBO-HYDRATE gm.	FAT gm.	INDIGESTIBLE RESIDUE			
							Lignin gm.	Hemi-cellulose gm.	Cellulose gm.	Total gm.
Egg	10	447	25.8	14.2	4.7	26.6	5.45	4.67	3.66	13.78
Whole wheat cereal biscuit <sup>1</sup>	10	1102	24.3	28.8	38.0	38.0	19.10	43.20	18.00	80.30
Locally made whole wheat bread, 5% non-fat milk solids	10	731	25.8	18.6	20.0	24.5	16.90	22.60	11.30	50.80
Peeled wheat bread <sup>2</sup>	10	646	28.0	18.2	15.8	27.0	14.30	19.30	10.70	44.30
Lean white bread, I <sup>2</sup>	10	439	27.6	10.7	5.5	24.8	5.36	4.14	4.72	14.22
Peeled wheat bread	5	620	26.1	16.6	19.3	25.7	13.40	18.40	9.98	41.78
Peeled wheat bread, high vitamin yeast	5	609	25.0	17.6	20.9	25.9	13.40	22.9	13.19	49.49
White bread, 5% non-fat milk solids	5	512	24.4	10.9	4.6	17.3	6.02	4.75	8.28	19.05
Lean white bread, II	5	553	22.3	11.6	3.7	24.7	6.49	5.06	5.74	17.29

<sup>1</sup> Shredded Wheat Biscuit of the National Biscuit Company.

<sup>2</sup> Eaten with extra B vitamins.

TABLE 3  
*Average daily intake in grams and apparent digestibility calculated as per cent of intake.*

TEST FOOD	NO. OF SUBJECTS	TEST FOOD	ASH		CARBO-HYDRATE		FAT		INDIGESTIBLE RESIDUE	
			In-take gm.	App. dig. %	In-take gm.	App. dig. %	In-take gm.	App. dig. %	In-take gm.	App. dig. %
Egg	10	230	6.47	63.5	302	99.7	151	97.0	3.47	29.2
Whole wheat cereal biscuit <sup>1</sup>	10	254	8.95	46.4	270	97.6	140	95.7	25.87	48.2
Locally made whole wheat bread, 5% non-fat milk solids	10	281	10.57	70.5	243	98.7	144	97.1	16.41	47.0
Peeled wheat bread <sup>2</sup>	10	282	11.45	73.5	243	99.1	158	97.1	18.48	59.9
Lean white bread, I <sup>2</sup>	10	337	11.31	84.4	270	99.7	171	97.4	9.48	75.4
Peeled wheat bread, high vitamin yeast	5	284	11.48	74.4	242	98.5	145	97.0	18.57	55.0
Peeled wheat bread	5	279	11.30	75.3	231	98.6	147	97.1	18.38	61.7
White bread, 5% non-fat milk solids	5	319	11.31	83.6	269	99.8	152	97.0	9.81	68.1
Lean white bread, II	5	327	10.70	62.1	267	99.8	143	97.2	10.90	76.3

<sup>1</sup> Shredded Wheat Biscuit of the National Biscuit Company.

<sup>2</sup> Eaten with extra B vitamins.

a striking uniformity of mineral intake occurred. In view of this finding it is regrettable that time did not permit complete mineral balances to be made in this experiment.

It is evident that the absorption of fat and carbohydrate is excellent in all instances. It is thus obvious that the extra indigestible residue of the whole wheat or peeled wheat bread as contrasted with the white breads does not interfere with the absorption of these two major foodstuffs. This result is in agreement with those of Adolph and Mao-Yi Wu ('34) as regards the influence of fiber on the digestibility of protein in the absence of a laxative effect.

The percentages recorded in the last column represent the relative absorption or, perhaps better, the disappearance of the indigestible residue. In view of present ignorance concerning the chemistry and digestive fate of the residue components, these values can hardly be interpreted as indicating digestive utilization. The authors are not alone in finding apparently high values for "absorption" of "indigestible residue." For example, Heller and Wall ('40) reported coefficients of utilization of indigestible residue of 16 to 52% when cereal feeds were fed to rats. These values obtained by a modified Williams and Olmsted method may be compared with the range of 29 to 76% found in this experiment. For a more detailed discussion of this question and that of accuracy obtainable the former paper as well as that of Crampton and Maynard ('38) should be consulted.

An additional example of this phenomenon should be cited. Shepherd, Hummel and Macy ('40) have reported the disappearance of these same constituents in connection with their studies on children. The values recorded range from 59 to 91% of cellulose and 37 to 73% of the hemicellulose ingested. It is certain that the final solution of this problem must await more accurate chemical characterization and investigation of the fate of these materials in the intestinal tract.

Special attention is drawn to the parallelism between the nitrogen and the total indigestible residue in the feces, as related to wet weight of feces, illustrated in figure 1. On each

curve the lower points pertain to the values for the white breads, the middle group of points to those for the whole wheat breads, and the extreme upper right-hand group for the whole wheat cereal biscuit. This parallelism is strongly confirmatory of the concept which originated with the Voit school of physiologists that the nitrogen content of the feces

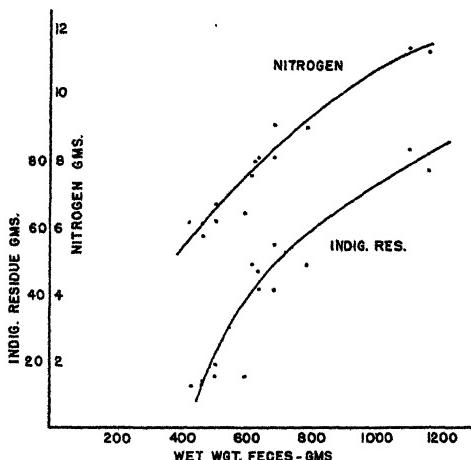


Fig. 1 Parallelism between total indigestible residue and fecal nitrogen in their relation to total moist weight of feces.

is more closely related to the endogenous (alimentary) products than to the nitrogen content of the foods. In this conception indigestible residue is related to these endogenous products as an excitant for the production of increased alimentary secretion. The difficulties attendant to the exact separation of food N from alimentary N in the feces are not wholly overcome by use of the biological value method, as all students of this problem agree.

#### SUMMARY

The effect of white and whole wheat breads and shredded wheat on the apparent digestibility of fat, carbohydrate and "indigestible residue" has been determined with ten men as subjects.

The apparent digestibility of carbohydrates varied from 97.6 to 99.8% and of fat from 95.7 to 97.4%. No significant difference between the results obtained with white breads and whole wheat bread was found.

It may be concluded that under the conditions of these experiments in which the diet furnished approximately adequate calories for maintenance of weight of each subject, the higher "indigestible residue" of the whole wheat products does not interfere with the digestion and absorption of carbohydrate and fat.

The "indigestible residue" exhibited an apparent digestibility of 29.2 to 76.3%. The white breads gave the higher values, 68 to 76%, and the whole wheat products the somewhat lower values of 55 to 62%.

#### LITERATURE CITED

- ADOLPH, W. H., AND MAO-YI WU 1934 The influence of roughage on protein digestibility. *J. Nutrition*, vol. 7, p. 381.
- CRAMPTON, E. W., AND L. A. MAYNARD 1938 The relation of cellulose and lignin content to the nutritive value of animal feeds. *J. Nutrition*, vol. 15, p. 383.
- HELLER, V. G., AND R. WALL 1940 The indigestible carbohydrates of feeds. *J. Nutrition*, vol. 19, p. 141.
- MURLIN, J. R., M. E. MARSHALL AND C. D. KOCHAKIAN 1941 Digestibility and biological value of whole wheat breads as compared with white bread. *J. Nutrition*, vol. 22, no. 6, pp. 573-588.
- SAXON, G. J. 1914 A method for the determination of the total fats of undried feces and other moist masses. *J. Biol. Chem.*, vol. 17, p. 99.
- SHAFFER, P. A., AND M. SOMOGYI 1933 Copper-iodometric reagents for sugar determination. *J. Biol. Chem.*, vol. 100, p. 695.
- SHEPHERD, M. L., F. C. HUMMEL AND I. G. MACY 1940 Influence of raw banana and apple upon disappearance of complex carbohydrates from the alimentary tracts of normal children. *Am. J. Digestive Diseases*, vol. 7, p. 248.
- SOMOGYI, M. 1930 A method for the preparation of blood filtrates for the determination of sugar. *J. Biol. Chem.*, vol. 86, p. 655.
- WILLIAMS, R. D., AND W. H. OLMSSTED 1935 A biochemical method for determining indigestible residue (crude fiber) in feces: lignin, cellulose, and non-water-soluble hemicelluloses. *J. Biol. Chem.*, vol. 108, p. 653.

# DARK ADAPTOMETER AND BLOOD VITAMIN A MEASUREMENTS IN A NORTH CAROLINA NUTRITION SURVEY

M. E. YARBROUGH<sup>1</sup> AND W. J. DANN

*Department of Physiology and Pharmacology,  
Duke University School of Medicine, Durham, North Carolina*

ONE FIGURE

(Received for publication July 21, 1941)

The purpose of this investigation was to measure the amount of carotene and vitamin A in the blood of subjects whose visual threshold had been determined with the Hecht and Shlaer dark adaptometer. A number of instruments have been designed for the purpose of measuring visual adaptation in dim light following exposure to bright light as a test for the state of vitamin A nutrition in man (Jeans and Zentmire, '34; Edmund and Clemmesen, '36; Feldman, '36; Jeans et al., '37; Hecht and Shlaer, '38; Wald, Jeghers and Arminio, '38; Pett, '39; Haig and Lewis, '39; Steele, '40). The above-mentioned dark adaptometer is one of the most promising of these since it permits determination of the visual threshold throughout the process of dark adaptation, and it meets certain specifications enumerated by the authors as essential for measurement of visual thresholds, that have not been met in earlier instruments.

During the past 15 years much evidence has been obtained to establish a definite relationship between night blindness and vitamin A nutrition. Holm ('25) devised a jumping test for

<sup>1</sup> From a thesis submitted by M. E. Yarbrough in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Duke University.

vision in rats and showed that night blindness is an early symptom of vitamin A deficiency, and Fridericia and Holm ('25) found that the rate of regeneration of visual purple in the retinas of rats is much slower in those deprived of vitamin A than in normal animals. In 1935 Wald demonstrated that vitamin A combines in the retina with a protein to form visual purple, which in the presence of light changes to visual yellow. A part of the visual yellow is resynthesized to visual purple and the other part decomposes into vitamin A and a protein. Some of the vitamin A is destroyed and must be replaced by vitamin A from the blood. In avitaminosis-A this cycle is retarded, the visual threshold is raised and night blindness results. From these findings it seemed logical to use the measurement of visual adaptation as a test for early stages of vitamin A deficiency in man, and the various above-mentioned instruments were devised for measuring visual adaptation and making such tests.

If night blindness is due to a lack of visual purple in the retina which is in turn due to a lack of vitamin A supplied from the blood it seems reasonable to assume that the readings from any instrument that is a satisfactory indicator of vitamin A deficiency should correlate with blood vitamin A. In this investigation the Hecht and Shlaer dark adaptometer was used for measuring the visual threshold levels of subjects whose blood vitamin A and carotene had been determined, and the degree of correlation between the results of the two tests was calculated.

#### EXPERIMENTAL

The subjects of this study were those included in a Nutrition Survey<sup>2</sup> being made of a typical North Carolina rural mill village. About 60% of the total population, selected without bias as to economic and social grouping, was examined. The ages ranged from 6 to 68 years for the adaptometer measurements, and from 5 months to 68 years for the blood vitamin A

<sup>2</sup> The Cooperative Nutrition Survey in North Carolina is sponsored by the North Carolina State Board of Health in cooperation with the Rockefeller Foundation and Duke University Medical School. The first community survey was in Chatham County.

and carotene determinations. Both tests were made on 154 subjects and the blood tests alone on forty-three additional subjects. These subjects will be referred to as the "nutrition survey group".

Before examining the nutrition survey subjects, determinations were made on a "test group" composed of graduate students and members of the medical school staff. Since this group numbered only sixteen it was not satisfactory for establishing normal values, but the values thus obtained served as a check on the instrument and on the technique of the operator when compared with those reported as normal by Hecht and Mandelbaum ('39) and Isaacs et al. ('40).

*Vitamin A and carotene in the blood.* Non-fasting blood samples were used throughout this investigation. These samples were taken within 24 hours of the adaptometer test in 34.4% of the cases, within 10 days in 90.4%, and the remainder within the main survey period of 6 weeks. The following evidence indicates that this procedure is justifiable. Kimble ('39) has reported that ingestion of 250,000 I.U. of vitamin A by each of two individuals more than doubled the plasma vitamin A in 2 hours, but that ordinary meals did not cause a measurable increase in either the vitamin A or the carotene within 2 to 6 hours. If no vitamin A concentrate was taken with the meal, then she found that fasting and post-prandial samples gave the same levels for vitamin A and carotene. We have extended this observation by administration of 20,000 I.U. of vitamin A together with feeding a light breakfast devoid of vitamin A rich foods to each of sixteen normal adult subjects, followed by measurement of the plasma level at intervals. The highest level of vitamin A in the plasma was generally found at the second or third hour after the meal and the average increase over the fasting level was 30%.

The amount of vitamin A ingested with these experimental meals may be compared with the intake of the nutrition survey subjects. Their average daily intake was 3,500 I.U., and only seven ingested more than 10,000 I.U. daily, the highest intake being 14,500 I.U. This intake was largely in the form

of carotene which is known to be less efficiently absorbed from the intestine than the colorless vitamin A. From these figures we conclude that the ingestion of vitamin A by the nutrition survey subjects at any single meal was not large enough to cause an increase in vitamin A of the plasma greater than the error of the determination. This conclusion is strengthened by the observation that for the twelve subjects whose vitamin A intake was greatest, the mean vitamin A content of the plasma was 52 I.U. per 100 ml., compared with 51 I.U. for the entire group of 154.

The method employed for determining the vitamin A and carotene of the plasma is essentially that of May et al. ('40). The datum  $L_{440}$  was used as a measure of the carotene, and  $L_{620}$  as a measure of vitamin A. From  $L_{440}$  the carotene content of the plasma in micrograms per 100 ml. plasma was obtained on multiplying by the factor 694. From  $L_{620}$  allowance for the blue color produced by the carotene and for meniscus error of the solution in the open colorimeter cell was first made by calculating  $L_{620}$  (corrected) =  $1.43 L_{620} - 0.14 L_{440}$ . The determination of the constant 1.43 will be described in a later article; the constant 0.14 was established by Dann and Evelyn ('38). The corrected  $L_{620}$  was then multiplied by the factor 600 to convert it to International Units of vitamin A per 100 ml. plasma.

The mean and standard deviation of the determinations for both the test group and the nutrition survey group are given in table 1. Table 2 gives the normal values reported by other investigators who have used a similar method of extraction and a photoelectric colorimeter for color measurements. It is seen that the carotene values for both the test group and the nutrition survey group, and the vitamin A figure of the former, compare favorably with the other recorded figures, but the blood vitamin A value of the nutrition survey group is slightly below these reported for the normals. This finding accords well with the data of the dietary survey of these subjects, which showed that no food particularly rich in vitamin A was

used by them and that the carotene of green vegetables provided the greater part of the vitamin A value of their diets.

*Method of dietary survey.* This survey was carried out by the staff of the Cooperative Nutrition Survey in North Carolina, and a detailed account will be published elsewhere. Each subject was provided with a form for recording all foods ingested during a period of 1 week, and instructed how to fill it

TABLE 1  
*Test Group*      *Nutrition Survey Group*

MEASUREMENT MADE	NO. SUBJECTS	MEAN AND STANDARD ERROR	RANGE	NO. SUBJECTS	MEAN AND STANDARD ERROR	RANGE
Visual threshold (log threshold- $\mu$ l.)	16	3.012 $\pm 0.037$	2.799 to 3.264	154	2.936 $\pm 0.021$	2.337 to 3.800
Blood carotene ( $\mu$ g. per 100 ml. plasma)	16	183 $\pm 18.4$	96 to 327	197	131 $\pm 4.232$	34 to 422
Blood vitamin A (I.U. per 100 ml. plasma)	16	71 $\pm 3.3$	44 to 88	197	51 $\pm 1.23$	7 to 101

TABLE 2  
*Carotene and vitamin A values of human blood.*

	CAROTENE $\mu$ G. PER 100 ML. PLASMA	VITAMIN A I.U. PER 100 ML. PLASMA
Kimble ('39)	166 for men range 50 to 300  186 for women range 90 to 340	127 for men range 89 to 198  91 for women range 67 to 169
May et al. ('40)	135 to 525 children 2 to 12 yrs.	49 to 164
Caveness et al. ('41)	Mean 230 stand. dev. 103	Mean 68 stand. dev. 16.5
Sobotka ('41)	100 to 250	60 to 80
Murrill et al. ('41)	Mean 213 stand. dev. 72	Mean 93 stand. dev. 15
Test Group	Mean 183 stand. dev. 74	Mean 71 stand. dev. 13.2
Nutrition Survey Group	Mean 131.2 stand. dev. 59.4	Mean 51 stand. dev. 17.2

out. He or she was seen daily during the recording period and the record checked for accuracy of the entries made. Children's records were kept by their parents. The only foods providing significant amounts of vitamin A were found to be eggs and green vegetables, and for a few individuals, milk was also important. Amounts of these foods ingested were recorded as number of eggs, tablespoonfuls of vegetables (average weight of tablespoonful being taken from a number of weighings made during the survey), and glasses of milk, at 8 ounces to the glass. The vitamin A values of these food-stuffs were taken from the tables of Sherman and Lanford ('40).

*Adaptometer readings.* According to Wald et al. ('38) and Hecht and Mandelbaum ('40) the most exact index of the level of vitamin A nutrition which can be obtained by dark adaptation measurements is the rod threshold determined after the subject has been in the dark long enough for virtual completion of dark adaptation. We therefore measured the rod threshold for a retinal area 3 degrees in diameter situated 7 degrees nasally for flashes of violet light 0.2 second in duration. This is the procedure of Hecht and Mandelbaum except that we dispensed with the initial light-adaptation. The subject entered the dark room and the first threshold measurement was made after 28 minutes; subsequent measurements were made at 3-minute intervals until the 40-minute reading. The 40-minute reading was used for the correlation studies.

The mean and standard deviation of the visual thresholds are given in table 1. The thresholds of the test group ranged from 2.78 to 3.26 (log micro-microlamberts). These limits are within the narrow range that includes 80% of the normals of Hecht and Mandelbaum ('39, '40), and well within the range of 1.0 log unit of Isaacs et al. ('40). The range of thresholds among the nutrition survey group is wider, from 2.30 to 3.80. With the exception of three observed thresholds at 3.70 to 3.80, all the remainder may be adjudged normal by the criteria of the two groups of authors just mentioned. It is of interest that the remaining thresholds after discarding these three are

distributed in a way which does not depart significantly from the form of the normal law of error, as judged by calculation of the *g* statistics of Fisher ('38). But if these three thresholds are included then the *g* statistics indicate a significant departure from the normal distribution toward skewness.

The graphical representation of the frequency distribution of the nutrition survey group is given in figure 1; as is shown there, the total spread of the final rod threshold values is

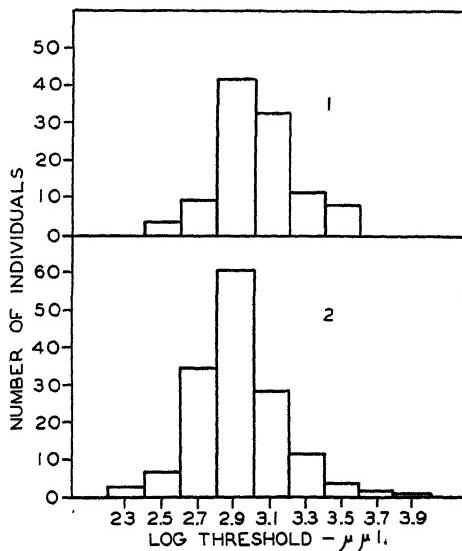


Fig. 1 Distribution of the rod threshold results of (1) 110 normal individuals by Hecht and Mandelbaum and (2) 154 nutrition survey subjects.

1.6 log units. Figure 1 also includes for comparison the distribution of the threshold readings from 110 normal subjects of Hecht and Mandelbaum ('39), with a total range of 1.0 log unit. This same spread of results was found by Isaacs et al. ('40) for sixty-eight normal subjects. Although the readings on the nutrition survey group show a wider range, 96.7% of them are below 3.50 log unit which is the highest value of the Hecht and Mandelbaum subjects. By the standards of these

investigators, it appears that 96.7% of the nutrition survey group subjects are normal.

*Correlation coefficients.* The correlation coefficients were found to be: (1) for dark adaptation threshold with plasma carotene,  $r = +0.158$ , and (2) for dark adaptation threshold with plasma vitamin A,  $r = +0.131$ . For 154 pairs of figures the correlation coefficient must differ from zero by more than 0.157 to reach the 5% level of significance (Snedecor, '40).

#### DISCUSSION

Assuming that hemeralopia is due to a vitamin A deficiency, it might be expected that the results obtained from a reliable instrument would be correlated with the amount of vitamin A in the blood. Experimental evidence tested on this assumption has been presented by workers using instruments other than the Hecht and Shlaer dark adaptometer.

Steininger et al. ('39) and Caveness et al. ('41) used the biophotometer for dark adaptation determinations. The latter calculated the correlation coefficients and found only slight correlation between blood vitamin A and biophotometer readings; the former reported no correlation from inspection of the results obtained. The "dark adapted visual threshold" readings were made with the biophotometer 15 minutes after exposure to bright light, not at the time when the final rod threshold had been reached but when it was still changing. The conclusion was drawn by Caveness et al. "that the biophotometer is unlikely to prove a suitable instrument for measuring with any precision the degree of vitamin A under-nutrition of the human subject".

Pett and LePage ('40) reported a definite correlation of blood vitamin A with the visual test developed by Pett ('39). This is a qualitative test and was made within 1 minute after light adaptation, again at a time when the visual threshold is changing very rapidly. Their method for measuring vitamin A in the blood is also open to objections.

In this investigation no correlation was found between either the Hecht and Shlaer dark adaptometer readings and blood

carotene figures or between the adaptometer readings and blood vitamin A figures. These results make it necessary to conclude that the Hecht and Shlaer dark adaptometer is not reliable for detecting mild avitaminosis A by means of a single test. This conclusion seems to be substantiated by the observation that the nutrition survey group gave blood vitamin A results below normal while 96.7% of the dark adaptometer readings were normal according to the criterion of Hecht and Mandelbaum.

In a recent article appearing since our observations were made, Steven and Wald ('41) have suggested that "a more searching criterion than low or high threshold is needed to estimate vitamin A deficiency"; They suggest instead the "vitamin A-labile threshold", which is "one that, whatever its initial value, is lowered at least 0.3 logarithmic unit within 2 weeks of regular vitamin A supplementation". Our experience with hospitalized subjects not reported in this paper fully confirms this view, and we have been using the "vitamin A-labile threshold" as the criterion for deficiency for some time.

Unfortunately the necessity both of treating the subject with vitamin A following the first measurement of visual threshold, and of taking a second measurement, sharply limits the applications of the test. It renders the test impracticable for nutritional surveys of the type provided by our subjects, and it also makes it impossible by this test to select a group of slightly vitamin A-deficient subjects for other observations, since they can only be detected by being cured.

#### SUMMARY AND CONCLUSIONS

Our results lead to the conclusions:

1. A single measurement of the visual threshold cannot be a reliable indicator of mild avitaminosis A. This is in agreement with the conclusion of Steven and Wald, who suggest the necessity of determining also the stability or lability of the threshold.

2. The level of vitamin A in the blood seems to be the most promising method of detecting mild avitaminosis A by means of a single test, although more data are needed to establish the range of normal and abnormal values.

3. The blood vitamin A and carotene levels are somewhat lower for the rural population tested than have been reported for other groups of normal individuals here and elsewhere.

Our thanks are due to the Duke University Research Council for a grant, and to Dr. D. F. Milam (Director) and the staff of the Cooperative Nutrition Survey in North Carolina, for their cooperation in carrying out this study.

#### LITERATURE CITED

- CAVENESS, H. L., G. H. SATTERFIELD AND W. J. DANN 1941 Correlation of the results of the biophotometer test with the vitamin A content of human blood. *Arch. Ophthalmol.*, vol. 25, p. 827.
- DANN, W. J., AND K. A. EVELYN 1938 The determination of vitamin A with the photoelectric colorimeter. *Biochem. J.*, vol. 32, p. 1008.
- EDMUND, C., AND S. CLEMMESSEN 1936 On deficiency of A vitamin and visual dys-adaptation, 92 pp. Copenhagen and Oxford.
- FELDMAN, J. B. 1936 Dark adaptation as a clinical test. *Arch. Ophthalmol.*, vol. NS 15, p. 1004.
- FISHER, R. A. 1938 Statistical methods for research workers, 7th ed. 356 pp. Edinburgh.
- FRIDERICIA, L. S., AND E. HOLM 1925 Experimental contribution to the study of the relation between night blindness and malnutrition. *Am. J. Physiol.*, vol. 73, p. 63.
- HAIG, C., AND J. M. LEWIS 1939 Simple method of measuring brightness threshold of dark adapted eye at all ages. *Proc. Soc. Exp. Biol. and Med.*, vol. 41, p. 415.
- HECHT, S., AND J. MANDELBAUM 1939 The relation between vitamin A and dark adaptation. *J. Amer. Med. Ass.*, vol. 112, p. 1910.
- 1940 Dark adaptation and experimental human vitamin A deficiency. *Am. J. Physiol.*, vol. 130, p. 651.
- HECHT, S., AND S. SHLAER 1938 An adaptometer for measuring human dark adaptation. *J. Opt. Soc. Am.*, vol. 28, p. 269.
- HOLM, E. 1925 Demonstration of hemeralopia in rats nourished on food devoid of fat-soluble-A-vitamin. *Amer. J. Physiol.*, vol. 73, p. 79.
- ISAACS, B. L., F. T. JUNG AND A. C. IVY 1940 Clinical studies of vitamin A deficiency. *Arch. Ophthalmol.*, vol. NS 24, p. 698.
- JEANS, P. C., E. BLANCHARD AND Z. ZENTMIRE 1937 Dark adaptation and vitamin A. A new photometric technic. *J. Amer. Med. Ass.*, vol. 108, p. 451.

- JEANS, P. C., AND Z. ZENTMIRE 1934 A clinical method for determining moderate degrees of vitamin A deficiency. *J. Amer. Med. Ass.*, vol. 102, p. 892.
- KIMBLE, M. S. 1939 The photocolorimetric determination of vitamin A and carotene in human plasma. *J. Lab. and Clin. Med.*, vol. 24, p. 1055.
- MAY, C. D., K. D. BLACKFAN, J. F. McCREARY AND F. H. ALLEN 1940 Clinical studies of vitamin A in infants and children. *Am. J. Dis. Children*, vol. 59, p. 1167.
- MURRILL, W. A., P. B. HORTON, E. LEIBERMAN AND L. H. NEWBURGH 1941 Vitamin A and carotene metabolism in diabetics and normals. *J. Clin. Invest.*, vol. 20, p. 395.
- PETT, L. B. 1939 Vitamin A deficiency: its prevalence and importance as shown by a new test. *J. Lab. and Clin. Med.*, vol. 25, p. 149.
- PETT, L. B., AND G. A. LEPAGE 1940 Vitamin A deficiency. III. Blood analysis correlated with a visual test. *J. Biol. Chem.*, vol. 132, p. 585.
- SHERMAN, H. C., AND C. S. LANFORD 1940 *Essentials of Nutrition*. Macmillan, New York.
- SNEDECOR, G. W. 1940 *Statistical Methods*, 3rd edition. 422 pp. Ames.
- SOBOTKA, H. H. 1941 Personal communication.
- STEELE, E. J. P. 1940 Effect of vitamin A therapy estimated by a rapid optical test. *Lancet*, vol. 2, p. 205.
- STEININGER, G., L. J. ROBERTS AND S. BRENNER 1939 Vitamin A in the blood of normal adults. *J. Amer. Med. Ass.*, vol. 113, p. 2381.
- STEVEN, D., AND G. WALD 1941 Vitamin A deficiency: a field study in Newfoundland and Labrador. *J. Nutrition*, vol. 21, p. 461.
- WALD, G. 1935 Carotenoids and the visual cycle. *J. Gen. Physiol.*, vol. 19, p. 351.
- WALD, G., H. JEGHERS AND J. ARMINIO 1938 An experiment in human dietary night-blindness. *Am. J. Physiol.*, vol. 123, p. 732.



## MAGNESIUM STUDIES IN CALVES

### II. THE EFFECT OF MAGNESIUM SALTS AND VARIOUS NATURAL FEEDS UPON THE MAGNESIUM CONTENT OF THE BLOOD PLASMA<sup>1</sup>

C. F. HUFFMAN, C. L. CONLEY, C. C. LIGHTFOOT AND C. W. DUNCAN  
*Dairy Section and Chemistry Section of the Michigan Agricultural  
Experiment Station, East Lansing*

(Received for publication July 31, 1941)

In a previous paper Duncan, Huffman and Robinson ('35) reported that calves receiving whole milk as the sole ration, with or without the addition of various mineral and vitamin supplements, had low plasma magnesium values. Magnesium intakes as high as 14–16 mg. per kilogram of body weight failed to maintain normal plasma magnesium values on the above type of ration. With one exception (Herman, '36), these results have been substantiated by other workers (Knoop et al., '37, '39; Cave et al., '36; Wise et al., '39; Duckworth, '38–'39). The pathology associated with the low blood plasma magnesium values observed in our calves has been investigated by Moore, Hallman and Sholl ('38). The histologic changes associated with the low plasma magnesium values bear some similarities to those observed in rats (Greenberg, Anderson and Tufts, '36; Schrader, Prickett and Salmon, '37; Cramer, '32).

Many reports have been published on the metabolism of magnesium as affected by a variety of conditions but very few data are available on the relative utilization of the naturally occurring magnesium compounds in feedstuffs as compared to the utilization of the inorganic magnesium salts.

<sup>1</sup> Published with the permission of the Director of the Agricultural Experiment Station as Journal Article No. 535 (n.s.).

There has always been some controversy regarding the merits of minerals from organic and inorganic sources in nutrition, but generally speaking, basal rations inadequate in some minerals can usually be supplemented satisfactorily with inorganic sources insofar as the bovine is concerned. This paper reports a study of the comparative value of magnesium salts and various natural feeds upon magnesium metabolism of the growing bovine when fed as a supplement to whole milk ration, in the maintenance of a normal level of magnesium in the blood plasma.

#### EXPERIMENTAL

The precautions observed in the routine withdrawal of the blood samples, their disposition in the laboratory, and the methods used for the determination of plasma calcium, magnesium and inorganic phosphorus have been recorded (Duncan, Huffman and Robinson, '35). Plasma determinations were made each week and these values were averaged for the 30-day periods which are reported in the tables. The changes in plasma magnesium were often quicker and more pronounced than is indicated by the averaged values.

The basal ration consisted of whole milk, viosterol, iron, copper and manganese. In some instances either corn starch or a cereal breakfast food<sup>2</sup> was fed as an additional source of energy. This ration supplied 10-12 mg. of magnesium per kilogram of body weight. The basal ration was supplemented with either magnesium oxide (light), corn, alfalfa hay, alfalfa ash, corn gluten meal, or cane molasses. When the supplements were added to the basal ration, they were added as such and not on an equal calorie basis. With the exception of four calves, wood shavings were used as bedding. The calves also had free access to the shavings which were their only source of roughage. Four of the animals were placed on wooden slats and given free access to regenerated cellulose as their only source of roughage. Water was offered to the animals twice daily, and they received exercise in a dry lot except during inclement weather.

<sup>2</sup> Rice Krispies.

The viosterol was mixed with the milk at each feeding. No instances of vitamin A or D deficiencies were manifested. The mineral supplement consisted of C.P. ferrous sulfate, C.P. copper sulfate and C.P. manganese sulfate. The minerals were made up in solution and added to the milk at each feeding so that each kilogram of milk contained 16.9 mg. iron, 1.5 mg. copper and 1.3 mg. manganese. The magnesium supplements were also mixed with the milk and the amounts indicated in the tables represent the total magnesium intake. The calves were weighed in the morning before feeding at 10-day intervals.

#### RESULTS

Although the physiologic range of plasma magnesium may be comparatively wide (2.00 mg. per 100 cc., minimum to 2.80 mg., maximum), the mean magnesium value obtained from 107 normal calves from birth to 18 months of age has been found to be 2.41 mg., with a standard deviation of 0.38 mg. (Duncan, Lightfoot and Huffman, '38). Variations due to the season of the year have also been noted (Duncan, Lightfoot and Huffman, '40). The significance of the magnesium values in the tables are assessed on this basis.

*Magnesium oxide as a source of magnesium.* Although there were some individual variations in the amount of magnesium required to maintain normal plasma magnesium levels in this group, abnormally large amounts of magnesium were required in all cases. The results shown in table 1 for magnesium oxide are comparable to the results obtained from the use of the carbonate, chloride and phosphate. The efficiency of the citrate, silicate, sulfate and metallic magnesium was markedly reduced. More than thirty calves have been used to study the efficiency of the magnesium salts.

The plasma magnesium values for calf C 284 were subnormal during the basal feeding period. From 70 to 270 days of age the concentration of plasma magnesium returned to normal and remained within normal limits with an average daily magnesium intake of 53 mg. per kilogram of body weight.

TABLE 1  
*Effects of various magnesium intakes on the plasma magnesium in the calf.*

SUPPLEMENT USED	AGE	WEIGHT	MINERAL INTAKE			Mg INTAKE PER KILO BODY WEIGHT	BLOOD PLASMA		
			Ca	P	Mg		Ca	P	Mg
Basal C 284	days	kg.	grams per day			mg.	mg. per 100 cc.		
Basal C 284	30	39.8	5.3	3.8	0.49	12.3	12.8	8.17	2.04
	60	51.2	6.3	4.5	0.58	11.3	12.1	7.47	1.74
	69	59.5	6.4	4.6	0.59	9.9	12.0	7.33	1.64
MgO added	90	66.4	7.5	5.5	5.60	84.3	12.3	7.48	2.12
	120	82.9	8.8	6.7	5.84	70.4	11.4	7.35	2.39
	150	104.1	10.7	8.3	6.13	58.9	10.1	6.76	2.48
	180	124.1	11.4	8.8	6.19	49.9	8.7	7.27	2.41
	210	149.8	14.8	10.7	6.26	41.8	10.5	6.87	2.24
	240	180.6	14.7	10.5	6.18	34.2	11.8	8.33	2.30
	270	213.9	15.8	11.3	6.20	29.0	12.1	8.44	2.21
	300	227.3	15.7	11.2	7.88	34.7	11.8	7.86	1.94
	330	230.8	15.7	11.2	9.81	42.5	11.6	7.81	1.96
	360	245.5	16.5	11.8	11.15	45.4	11.4	7.79	1.98
	390	264.7	15.4	11.0	9.46	35.7	11.1	7.83	2.33
	412	268.6	11.7	8.4	7.23	26.9	11.4	9.02	1.77
MgO discontinued	417	262.3	14.2	10.2	1.31	5.0	10.1	8.39	1.75
	450	248.5	6.2	4.4	0.57	2.3	8.7	11.42	1.65
	455 <sup>1</sup>	239.1	6.2	4.4	0.57	2.4	6.5	16.26	1.40
Basal A 37	18	46.8	4.5	3.5	0.43	9.2	12.1	8.33	2.27
Molasses added	30 <sup>2</sup>	51.8	6.2	4.1	1.16	22.4	12.2	6.94	2.01
	60	64.5	7.2	4.9	1.25	19.4	11.6	7.07	1.97
	90	84.1	9.3	6.2	1.75	20.8	12.1	7.10	1.73
	120	100.9	10.0	7.1	2.16	21.4	10.8	7.96	1.72
Molasses discontinued, corn added	150 <sup>3</sup>	128.6	10.5	10.1	1.97	15.3	12.4	7.88	1.75
	180	161.8	10.6	10.2	2.20	13.6	11.9	7.75	2.12
	196	176.4	11.6	11.0	2.29	13.0	11.6	8.17	1.91
	210	187.7	12.3	11.7	2.92	15.6	11.9	8.12	2.11
	240	224.1	12.8	11.7	2.92	13.0	11.5	7.89	1.99
	270 <sup>4</sup>	247.3	12.3	11.7	2.92	11.8	11.6	7.87	2.12
Basal plus corn gluten meal A 35	30 <sup>5</sup>	52.7	4.9	3.6	0.46	8.7	11.3	8.14	2.39
	60	68.2	6.6	4.9	0.63	9.2	11.1	8.28	1.87
	90	90.9	7.4	5.6	0.73	8.0	11.4	7.07	1.86
	112	108.6	8.5	6.6	0.87	8.0	11.6	7.13	1.51
MgO added to above	142 <sup>6</sup>	133.2	9.0	7.0	2.12	15.9	11.5	8.93	1.61
	172	158.6	10.8	8.8	2.34	14.8	11.3	9.13	2.07
	202	185.5	11.8	9.5	2.47	13.3	12.2	8.26	1.93
	232 <sup>7</sup>	206.8	11.1	10.3	3.10	15.0	12.0	7.30	2.10
	249	219.1	11.5	10.3	3.11	14.2	11.9	7.53	1.52
	262 <sup>8</sup>	229.1	14.1	11.7	3.98	17.4	11.9	7.85	2.10
	292	253.2	14.1	11.7	3.98	15.7	11.7	6.99	1.82
	322 <sup>9</sup>	274.1	14.1	11.7	3.98	14.5	11.9	7.01	2.05

<sup>1</sup> Slaughtered.

<sup>2</sup> Increasing amounts from 0.5 to 0.9 pounds per day.

<sup>3</sup> 2 pounds per day to 196 days of age, 3 pounds thereafter.

<sup>4</sup> Changed to another experiment.

<sup>5</sup> Intake of CGM increased progressively from 0.1 to 1.0 pound per day to 112 days of age.

<sup>6</sup> 2 gm. MgO plus 2.0 pounds CGM per day to 232 days of age.

<sup>7</sup> 3 gm. MgO plus 2.5 pounds CGM per day to 262 days of age.

<sup>8</sup> 4 gm. MgO plus 3.0 pounds CGM per day to 322 days of age.

<sup>9</sup> Changed to another experiment.

The values from 270 to 412 days of age dropped below the normal range (less than 2.0 mg. per 100 cc.) when the average daily intake was reduced to 37 mg. per kilogram. The growth curve for C 284 was approximately normal until the magnesium supplement was discontinued at 413 days of age, after which time the body weight and the plasma magnesium values decreased markedly until the calf was slaughtered at 455 days of age.

Attention should be directed to the high plasma inorganic phosphorus values obtained during the latter stages of the experiment. These unusually high values have been observed in nearly all cases where the calves had been maintained on whole milk rations and were associated with pathologic kidneys. The most characteristic feature of the renal inadequacy was the retention of inorganic phosphorus in the blood. The failure of its excretion denoted an advanced and grave condition which usually terminated in death. Some degree of hypocalcemia was evident in many calves which had advanced renal damage but the concentration of plasma magnesium was not influenced to any extent.

*Corn as a source of magnesium.* Six calves were used to demonstrate the efficiency of corn when fed as a supplement to a whole milk ration in maintaining normal plasma magnesium levels. Data are presented in table 1 for calf A 37. At 19 days of age cane molasses was added to the whole milk ration. The addition of the molasses, which more than doubled the magnesium intake over the basal feeding period, was insufficient to meet the magnesium requirements. At 121 days of age, the molasses was omitted from the ration and 2 pounds of no. 2 yellow corn were substituted for the next 74-day period. This amount of corn was sufficient to increase the plasma magnesium to approximately normal levels. After 196 days of age, 3 pounds of corn per day were fed to maintain the plasma magnesium levels at the lower limits of the normal range until the calf was changed to another experiment at 270 days of age. The data obtained from the other calves in this group showed some individual variations but the results

of the group as a whole indicate that total magnesium intakes from 12 to 15 mg. per kilogram of body weight were sufficient to maintain normal growth and plasma magnesium values.

*Corn gluten meal as the source of magnesium.* Table 1 shows the effect of supplementing the whole milk ration of A 35 with corn gluten meal. The ingestion of from 0.1 to 1.0 pound per day in addition to the whole milk was ineffective in maintaining the plasma magnesium at normal levels. When small amounts of magnesium oxide were added to the ration to increase the total magnesium intake to 13-18 mg. per kilogram of body weight, the corn gluten meal then supplied the needed requirement to maintain normal plasma magnesium values. These small amounts of magnesium oxide were not effective, however, without the corn gluten meal. The appetite of the calves for corn gluten meal was quite poor and some difficulty was encountered in getting them to consume large amounts.

*Alfalfa hay as a source of magnesium.* Four calves were fed alfalfa hay as a supplement to the basal ration as the source of magnesium. The experimental data for calf C 401 are presented in table 2. This experiment may be divided conveniently into three periods, from birth to 180 days, from 180 to 330 days, and from 330 to 582 days of age during which the magnesium intakes averaged 30 mg., 16 mg., and 10 mg. per kilogram of body weight respectively. The results obtained with this calf and others in the group show that even the smallest supplement of hay per kilogram provided sufficient magnesium to meet the requirement for the maintenance of normal levels of plasma magnesium.

*Alfalfa ash as the source of magnesium.* Table 2 shows the results of supplementing the basal ration of calf C 273 with alfalfa ash. The efficiency of a small amount of alfalfa hay in maintaining normal plasma levels suggested the idea of destroying the organic material present in the hay and feeding equivalent amounts of the ash. A large amount of alfalfa was, therefore, burned in the air, and the ash was collected and fed as a supplement to the basal ration. The results indicate that 30-40 mg. of magnesium per kilogram of body weight

TABLE 2

*Effects of magnesium in alfalfa hay and alfalfa ash on the plasma magnesium in the calf.*

SUPPLEMENT USED	AGE	WEIGHT	MINERAL INTAKE			Mg INTAKE PER KILO BODY WEIGHT	BLOOD PLASMA		
			Ca	P	Mg		Ca	P	Mg
Basal plus alfalfa hay	days	kg.	grams per day			mg.	mg. per 100 cc.		
	30 <sup>1</sup>	59.0	9.1	4.2	1.36	23.0	11.2	5.04	2.85
	60	70.3	18.3	5.2	2.20	31.3	11.0	6.06	2.29
	90	87.5	14.1	5.8	2.27	25.9	12.5	7.04	2.20
	120	105.2	20.7	8.3	3.58	34.0	12.4	7.73	2.23
	150	118.8	24.4	8.9	4.29	36.1	12.5	8.98	2.67
	180 <sup>2</sup>	152.4	25.3	10.2	4.69	30.8	11.9	8.27	2.78
	210	174.6	18.1	9.6	3.19	18.3	11.4	8.84	2.44
	240	198.2	15.0	9.9	2.76	13.9	11.5	8.44	2.12
	270	224.5	19.7	10.5	3.82	17.0	11.4	9.26	2.23
	300	257.6	21.9	10.7	4.31	16.7	11.7	8.50	2.43
	330 <sup>3</sup>	279.9	18.6	10.3	3.57	12.8	11.9	7.80	2.00
	360	297.6	18.3	10.3	3.49	11.7	11.6	8.63	2.11
	390	317.5	18.3	10.3	3.49	11.0	11.4	8.83	2.50
	420	343.4	16.2	10.3	3.11	9.1	11.3	8.16	2.41
	450 <sup>4</sup>	357.2	15.6	10.3	2.88	8.1	11.4	8.72	2.09
	462	363.2	17.4	11.1	3.29	9.1	11.2	8.36	1.95
	492	389.1	20.3	10.6	4.58	11.8	11.0	7.99	2.61
	522	394.5	20.8	10.5	3.64	9.2	11.4	7.74	2.75
	552	411.8	20.4	10.5	3.49	8.5	11.2	7.71	3.07
	582	419.1	20.9	10.7	4.63	11.0	11.1	8.34	2.56
Basal C 273	30	46.1	5.6	4.0	0.52	11.3	11.9	7.21	2.14
	60	59.8	7.0	5.0	0.65	10.9	12.2	7.95	2.00
	90	78.2	8.6	6.3	0.87	11.1	12.3	8.00	1.81
	120	98.5	10.1	7.5	1.04	10.6	12.2	8.05	1.81
	134	114.5	10.6	7.8	1.04	9.1	11.2	8.65	1.61
	150	112.5	25.0	9.4	4.35	38.1	11.2	8.52	2.16
Alfalfa ash added	180	148.2	25.1	9.6	4.43	29.9	11.4	8.41	2.31
	210	165.0	25.2	10.0	4.62	28.0	11.1	8.48	1.85
	240	187.3	39.3	12.2	8.06	43.0	11.2	8.25	1.78
	270	210.6	41.2	13.3	8.31	39.5	11.2	8.39	1.96
	300	245.2	43.7	13.9	7.93	32.3	11.3	8.18	1.91
	330	267.4	43.6	13.8	7.89	29.5	11.1	8.72	1.61
	360	288.8	44.4	14.9	8.02	27.8	11.2	8.36	1.46
Ash discontinued. MgO added	366	295.9	16.8	12.0	1.55	5.2	10.2	6.87	1.38
	390	305.8	16.8	12.0	11.20	36.6	10.7	8.21	1.71
	420	318.6	17.2	12.3	11.24	35.3	10.6	8.90	2.25
	450	329.1	17.9	12.8	11.30	34.3	10.4	9.45	2.29
	480	358.6	18.0	12.9	11.31	31.5	10.5	8.57	2.19
	490 <sup>5</sup>	368.2	18.9	13.6	11.39	31.0	10.6	8.34	2.08

<sup>1</sup> Intake of alfalfa increased progressively from 0.4 to 2.0 pounds per day to 180 days of age.<sup>2</sup> Variable amounts of alfalfa from 0.6 to 1.5 pounds per day to 330 days of age.<sup>3</sup> 1.0 pound alfalfa per day to 450 days of age.<sup>4</sup> 1.5 pounds alfalfa per day to 582 days of age. Changed to another experiment.<sup>5</sup> Changed to another experiment.

in the form of alfalfa ash were not as effective as the same amount of magnesium in alfalfa hay. Intakes of 28-38 mg. per kilogram were sufficient to maintain normal plasma magnesium values for a short time but similar intakes failed to maintain the normal level for extended periods. The feeding of an equivalent amount of magnesium in the form of the oxide restored the plasma magnesium to normal levels. In three other calves, the ingestion of more than 38 mg. of magnesium per kilogram per day in the form of alfalfa ash was insufficient to maintain the level of plasma magnesium within the normal range.

#### DISCUSSION

The results of this investigation indicate that the utilization of magnesium by growing calves is more efficient when magnesium is furnished by natural feeds than by magnesium salts. The low plasma magnesium values and the symptoms observed following the long-continued feeding of a basal ration of whole milk, viosterol, iron, copper and manganese were prevented by supplementing the basal ration with either magnesium carbonate, oxide, phosphate or the chloride. An important observation in this work, however, was the large amount of dietary magnesium that was required to maintain normal plasma magnesium levels when magnesium salts were used as supplements. The feeding of 20-30 mg. of additional magnesium, bringing the total intake up to 30-40 mg. per kilogram, was required. The results of these experiments confirm those of Blakemore and Stewart ('34-'35), Allcroft and Green ('34), and Knoop et al. ('39) in showing that the feeding of magnesium salts to calves does not raise the plasma magnesium level above normal, contrary to the observations reported by Cunningham ('33, '34) on sheep and cows, and by Johnson, Palmer and Nelson ('40) on sheep.

When yellow corn was fed as a supplement to the basal ration, the total intake of 12 to 15 mg. of magnesium per kilogram of body weight was sufficient to maintain normal plasma magnesium values. Even better results were obtained with

alfalfa hay but more than 30 mg. of additional magnesium per kilogram failed to maintain the normal level when alfalfa ash was fed as a supplement to the whole milk ration. The results obtained from feeding corn gluten meal alone were inconclusive but when additional amounts of magnesium oxide were added to raise the total magnesium intake above 13 mg. per kilogram, normal plasma magnesium values were obtained and the calves grew at a normal rate. These results suggest that the factor, or factors, responsible for the decrease in the amount of magnesium required to maintain normal plasma magnesium values is organic in nature and is present in natural feeds such as alfalfa, corn, and corn gluten meal.

The results obtained from feeding calves a whole milk ration supplemented with cane molasses are contrary to those reported by Cunningham ('34) and Eveleth and Eveleth ('35) who stated that a drench of crude molasses or glucose elevated the serum magnesium in sheep and pigs. In all cases, the addition of molasses to the ration of young calves failed to maintain normal plasma magnesium values. The hypomagnesemia resulting from the whole milk ration appeared to be intensified upon the addition of the molasses. Since milk is high in lactose and molasses is high in sucrose, it does not appear logical that the beneficial results obtained from the feeding of alfalfa, corn or corn gluten meal can be explained on the basis of the sugar content of either supplement.

The failure of 10-12 mg. of magnesium per kilogram of body weight to maintain normal plasma magnesium values in calves fed whole milk as the sole source of magnesium is of additional interest in view of the observation of Greenberg and Tufts ('38) that the magnesium requirement of the rat is satisfied by an intake of 5 mg. per 100 gm. of food. These investigators also reported that the minimal requirement is raised to some extent by a high calcium or low vitamin G intake. Milk is high in calcium which would appear to explain the results obtained with corn but would not account for the results obtained with alfalfa hay which contains from 1.5-2.0% calcium. Milk is also high in vitamin G (riboflavin) so that a deficiency

of this factor is probably not responsible for the higher magnesium requirement of the calf when milk is used as the sole ration. Other members of the B-complex are not entirely ruled out, however, although most of these factors are considered to be synthesized in the rumen of the ruminant (McElroy and Goss, '39). Day and Orent-Keiles ('39) reported that rats fed whole milk ad libitum, supplemented with thiamine, iron, copper and manganese failed to develop the symptoms of magnesium deficiency. The results with rats, however, are not comparable to the results obtained with calves because rats usually consume from two to three times as much milk per kilogram of body weight as do calves. Our results suggest that milk may be deficient in some factor necessary for normal magnesium metabolism in the young bovine.

The data presented in the tables indicate that the average daily intake of calcium and phosphorus and the calcium: phosphorus ratio of the whole milk ration alone or with the various supplements provided an adequate amount of these elements for the normal growth of young bovine. The vitamin D content of the milk and viosterol, plus exposure to direct sunlight, was sufficient to permit normal calcium and phosphorus metabolism and to maintain normal plasma calcium and inorganic phosphorus levels until the kidneys became pathologic. The calcium:magnesium ingestion ratios indicate that they are not the influencing factors in the maintenance of normal plasma values. The magnesium supplement in the form of alfalfa hay, corn or corn gluten meal had calcium:magnesium ingestion ratios varying from 3.5 to 6.5:1 which were satisfactory in maintaining normal plasma magnesium levels. The plasma calcium level was unaltered on both the high and low magnesium intakes. Hypomagnesemia in calves, therefore, cannot always be correlated with a low dietary intake of magnesium since it is unrelated, in a direct manner, with the magnesium content of the ration.

#### SUMMARY

A basal ration consisting of whole milk supplemented with iron, copper and manganese, with or without the addition of

starch or a carbohydrate rich cereal product always produced hypomagnesemia in calves.

The total intake of 12–15 mg. of magnesium per kilogram of body weight was sufficient to maintain normal plasma magnesium levels when small amounts of either corn, alfalfa hay or corn gluten meal were included in the basal ration.

A total intake of 30 to 40 mg. of magnesium per kilogram was required to maintain normal plasma values, when magnesium salts were added to the basal ration. The same amounts of magnesium in the form of alfalfa ash were less effective than the magnesium salts.

These data suggest that the utilization of magnesium by growing calves is more efficient when magnesium is furnished by natural feeds than when supplied by magnesium salts.

#### LITERATURE CITED

- ALLCROFT, W. M., AND H. H. GREEN 1934 Blood calcium and magnesium of the cow in health and disease. *Biochem. J.*, vol. 28, p. 2220.
- BLAKEMORE, F., AND J. STEWART 1934–1935 Studies on the magnesium contents of the blood of cows in lactation tetany districts. *Univ. Cambridge Inst. Animal Path.*, Fourth Rept., p. 103.
- CAVE, H. W., W. H. RIDDELL, J. S. HUGHES, C. H. WHITNAH AND H. F. LIENHARDT 1936 Factors influencing the mineral metabolism of dairy animals. *Kan. Agr. Exp. Sta. Rept.*, 1932–34, p. 71.
- CRAMER, W. 1932 Experimental production of kidney lesions by diet. *Lancet*, vol. 223, p. 174.
- CUNNINGHAM, I. J. 1933 Magnesium in animal diets. The influence of the level of dietary magnesium on the magnesium and calcium contents of the bones, the bodies, and the blood serum of rats. *New Zealand J. Sci. Tech.*, vol. 15, p. 191.
- 1934 Magnesium and calcium in the blood of sheep and cows. Variations in the levels of blood magnesium and calcium in sheep and dairy cows with supplementary feeding of magnesium. *New Zealand J. Sci. Tech.*, vol. 15, p. 414.
- DAY, H. G., AND E. ORENT-KEELLS 1939 Adequacy of cow milk as a source of magnesium for rats. *Proc. Soc. Exptl. Biol. and Med.*, vol. 40, p. 638.
- DUCKWORTH, J. 1938–1939 Magnesium in animal nutrition. *Nutrition Abst. and Reviews*, vol. 8, p. 841.
- DUNCAN, C. W., C. F. HUFFMAN AND C. S. ROBINSON 1935 Magnesium studies in calves. I. Tetany produced by a ration of milk or milk with various supplements. *J. Biol. Chem.*, vol. 108, p. 35.
- DUNCAN, C. W., C. C. LIGHTFOOT AND C. F. HUFFMAN 1938 Studies on the composition of bovine blood. I. The magnesium content of the blood plasma of the normal dairy calf. *J. Dairy Sci.*, vol. 21, p. 689.

- DUNCAN, C. W., C. C. LIGHTFOOT AND C. F. HUFFMAN 1940 Studies on the composition of bovine blood. II. Seasonal variations in the level of magnesium in the blood plasma of growing dairy calves. *J. Dairy Sci.*, vol. 23, p. 125.
- EVELETH, D. F., AND M. W. EVELETH 1935 Blood chemistry of swine. II. Further studies of blood changes following the ingestion of glucose. *J. Biol. Chem.*, vol. 111, p. 753.
- GREENBERG, D. M., C. E. ANDERSON AND E. V. TUFTS 1936 Pathological changes in the tissues of rats reared on diets low in magnesium. *J. Biol. Chem.*, vol. 114, p. xliii.
- GREENBERG, D. M., AND E. V. TUFTS 1938 The biochemistry of magnesium deficiency. II. The minimum magnesium requirement for growth, gestation, and lactation, and the effect of dietary calcium level thereon. *J. Biol. Chem.*, vol. 122, p. 715.
- HERMAN, H. A. 1936 Growth and development of dairy calves on a milk diet. *Mo. Agr. Exp. Sta. Research Bull.*, no. 245.
- JOHNSON, D. W., L. S. PALMER AND J. W. NELSON 1940 Failure of dietary magnesium imbalance to produce urinary calculi in wethers. *Vet. Med.*, vol. 35, p. 353.
- KNOOP, C. E., W. E. KRAUSS, T. S. SUTTON AND R. G. WASHBURN 1937 Iron and copper in a normal calf ration. *Ohio Agr. Exp. Sta. Bull.*, no. 579, p. 82.
- KNOOP, C. E., W. E. KRAUSS AND C. C. HAYDEN 1939 Magnesium and vitamin D relationships in calves fed mineralized milk. *J. Dairy Sci.*, vol. 22, p. 283.
- MCELROY, L. W., AND H. GOSS 1939 Report on four members of the vitamin B complex synthesized in the rumen of the sheep. *J. Biol. Chem.*, vol. 130, p. 437.
- MOORE, L. A., E. T. HALLMAN AND L. B. SHOLL 1938 Cardiovascular and other lesions in calves fed diets low in magnesium. *Arch. Path.*, vol. 26, p. 820.
- SCHRADER, G. A., C. O. PRICKETT AND W. D. SALMON 1937 Symptomatology and pathology of potassium and magnesium deficiencies in the rat. *J. Nutrition*, vol. 14, p. 85.
- WISE, G. H., W. E. PETERSON AND T. W. GULLICKSON 1939 Inadequacy of a whole milk ration for dairy calves as manifested in changes of blood composition and in other physiological disorders. *J. Dairy Sci.*, vol. 22, p. 559.

# THE RELATIVE ASSIMILATION OF FLUORINE FROM FLUORINE-BEARING MINERALS AND FOOD (TEA), AND FROM WATER AND FOOD<sup>1</sup>

MARGARET LAWRENZ AND H. H. MITCHELL

*Division of Animal Nutrition, University of Illinois, Urbana*

(Received for publication July 28, 1941)

The extent to which fluorine in food materials is absorbed from the alimentary tract of animals and retained in the tissues undoubtedly determines the extent of its physiological effect on the body. The assimilation of fluorine may be dependent upon the chemical combinations in which the fluorine occurs (Lawrenz, Mitchell and Ruth, '39 b, '40 a). Changes occur of nutrients with which it is associated in the diet (Lawrenz and Mitchell, '41), and the manner in which it is administered (Lawrenz, Mitchell and Ruth, '39 b, '40 a). Changes occurring in the animal body itself (Lawrenz, Mitchell and Ruth, '40 b) may also modify the assimilation of food fluorine. As the references cited indicate, this laboratory has made contributions to the various phases into which the problems relating to the assimilation of fluorine may be subdivided. The investigation to be reported in this paper represents an extension of this program, and is specifically concerned with a comparison at low levels of intake of the assimilation of fluorine in sodium fluoride, in calcium fluoride, in raw rock phosphate and in a high-fluorine food (green tea), and also of the fluorine in sodium fluoride administered in water and

<sup>1</sup> The authors gratefully acknowledge the assistance rendered to this investigation by the donation of funds by the Graduate School of the University of Illinois.

in food. The latter problem of the relative toxicity of fluorine in water and in food has previously been studied using cryolite as the source of fluorine (Lawrenz, Mitchell and Ruth, '39 b), but the practical importance of the problem, especially with reference to the significance of evidence of the toxicity of fluorine-bearing waters to an appraisal of safe concentrations of fluorine in foods, is such as to warrant further study.

The relative toxicities of different fluorine compounds are not constant, but vary with the amounts fed, such that the lower the concentration of fluorine in the diet the less variation there is in toxicity (Smith and Leverton, '34; Evans and Phillips, '39). Hence, at levels of fluorine of significance in human nutrition, marked differences in the toxicities of different fluorine compounds are not to be expected, and have not been observed in the comparison of cryolite (synthetic) with calcium fluoride (Lawrenz, Mitchell and Ruth, '39 a). However, not many comparisons of different fluorine compounds have been made at the low levels of intake of significance to the problem of the prevailing fluorine hazard in human nutrition.

This fluorine hazard is a resultant of the natural contamination of water supplies with fluorine, of the industrial contamination of certain foods with fluorine-containing insecticides, and of the natural occurrence of fluorine in foods. McClure ('39) has recently published a compilation of reported fluorine analyses on a considerable number of human foods, from which it may be inferred that, although this element is widely distributed among foods, it occurs in significant amounts only in teas and sea foods, with the exception of some analytical results on foods grown in certain fluorite areas, probably involving dust contamination.

The assimilability or toxicity of the fluorine in these naturally high-fluorine foods appears worthy of study. Reid ('36) has shown that the fluorine in Chinese teas is largely extracted in a 2% infusion of the leaves, and that the fluorine thus extracted when fed at comparatively high levels to young rats is capable of causing the white striations in the incisor teeth

characteristic of fluorosis. Lee and Nilson ('39) have reported experiments on the assimilation of fluorine by rats from canned salmon and mackerel, purporting to show that inorganic fluorine is three times as effective in producing tooth striations as the naturally-occurring fluorine in these sea foods, but the quantitative relations in the assimilation of fluorine from the various experimental diets used in this study can hardly be assessed in view of the wide differences in fluorine concentrations. Since in fish and mammals most of the fluorine is present in the skeleton, and since Ellis and Maynard ('36) have detected no difference in the toxicity of fluorine in sodium fluoride and in bone meal at low levels of intake, one would not expect the fluorine in fish to be appreciably less toxic than that in inorganic fluorides at nutritionally significant levels of intake. The marked difference that Coulson, Remington and Lynch ('35) noted in the assimilability of the arsenic in sea food (shrimp) and in inorganic arsenic would not be expected to hold for fluorine, but evidently the problem of fluorine assimilation from natural foods needs much further study by accurately quantitative methods.

#### PLAN OF EXPERIMENTS

*Experiment 1.* The first experiment involved a three-way comparison of calcium fluoride and sodium fluoride administered in water solution, and sodium fluoride administered in food. Thirty young rats, separated into trios on the basis of litter membership, sex and similarity of body weight, were used in this comparison. All rats received a basal diet of corn 20, starch 15.5, sucrose 5, dried yeast 8, dried skim milk 27, casein (Argentine) 8, butter 6, lard 6, cod liver oil 2, wheat germ oil 0.5 and salts<sup>2</sup> 2%. In each trio the rats were fed equal amounts of this food throughout the experiment. The first rat in each trio was given drinking water containing about 7.8 p.p.m. of fluorine in the form of calcium fluoride. The second rat in each trio received the same amount of drinking water containing an equal concentration of fluorine as sodium

<sup>2</sup> Wesson (Science, vol. 75, p. 339, 1932) salts with NaF omitted.

fluoride. The fluorine-bearing water was so apportioned to the rats as to provide approximately 9 p.p.m. of fluorine on the basis of the food consumed. The third rat in each trio received daily the same amount of the same sodium fluoride solution as was given to the second rat, but in this case it was added to the food.

In order to separate as widely as possible the time of ingestion of fluorine in water and fluorine in food for the purpose of securing the maximum effect of the difference in method of administration, all rats were given access to food only from 4 to 5:30 P.M. No water was allowed at this time except the small amounts required to moisten the food to prevent scattering (roughly equal to the weights of food). Access to water was permitted only between 9 and 11 A.M.

The rats were weighed weekly and, after 6 weeks of feeding, their teeth were examined weekly for the appearance of striations, using a jeweler's lens with a magnification of  $4\times$ . At the end of the fourteenth week of feeding the rats were all killed with ether, and the carcasses autoclaved and skeletonized in the customary manner. The bones, teeth and soft tissues were analyzed for fluorine by methods previously described (Lawrenz, Mitchell and Ruth, '39 a). Check rats selected from the litters providing experimental rats were sacrificed and analyzed for fluorine at the beginning of the experiment. The average content of fluorine on the live weight was 6.54 p.p.m.

*Experiment 2.* In the second experiment the assimilability by growing rats of the fluorine in green tea and in Tennessee raw rock phosphate was compared with that of the fluorine in sodium fluoride, all fluorine-bearing supplements being incorporated in the basal diet.

The tea was selected from three varieties, bought on the local market, for its high content of fluorine. Among the samples analyzed were a black tea,<sup>3</sup> containing 65 p.p.m. of fluorine, an orange pekoe,<sup>4</sup> found to contain 80 p.p.m. of fluor-

<sup>3</sup> Lipton.

<sup>4</sup> Tenderleaf.

ine, and a green tea<sup>5</sup> with 130 p.p.m. of fluorine. The latter variety was therefore chosen and a subsequent sample that was used in the experimental diets contained even more fluorine than the first, i.e., 178 p.p.m. The rock phosphate tested contained 3.90% of fluorine and 34.96% of calcium.

Twelve trios of litter mate rats were used in this three-way comparison, and check rats from each litter, totalling twelve, were analyzed and found to contain an average of 5.03 p.p.m. of fluorine. The first rat in each trio received a ration composed as follows: casein (Argentine) 20, salts<sup>6</sup> 4, cod liver oil 1.5, wheat germ oil 0.5, corn oil 8, dried yeast 8, corn 38, sugar 15, starch 4.5 and cellufLOUR 0.5%, with additions of CaHPO<sub>4</sub> and NaF in the proportions of 644.3 and 21.7 p.p.m., respectively.

The second rat in each trio received the same diet modified by the substitution of 5% of ground tea leaves for the starch and cellufLOUR and the omission of the CaHPO<sub>4</sub> and NaF.

The third rat in each trio received the same diet given to the first rat with the NaF replaced by 228.8 p.p.m. of rock phosphate and the proportion of CaHPO<sub>4</sub> reduced to 375 p.p.m.

The three experimental diets were designed to contain equal percentages of calcium and fluorine. The concentration of the latter element averaged 11.8 p.p.m. for each diet.

The rats in the second experiment were controlled and measured as were the rats in the first experiment, and, after each trio had consumed 750 gm. of food per rat, they were sacrificed, dissected and analyzed by the same procedures.

#### EXPERIMENTAL RESULTS

*Experiment 1.* The rats in this experiment grew as well as the rigorous conditions imposed would permit, gaining an average of about 1.5 gm. daily. The various diets promoted growth about equally well and no untoward symptoms were noted except the occurrence of slight amounts of blood in the

<sup>5</sup> Tenderleaf.

<sup>6</sup> See footnote 2, page 623.

urine of rat 276, receiving  $\text{CaF}_2$  in the drinking water, and rat 295, receiving  $\text{NaF}$  in the drinking water, on the fifth and fourth days of the test, respectively. A similar hematuria was observed in the feeding of cryolite to rats in the drinking water (Lawrenz, Mitchell and Ruth, '39 b).

Striations of the teeth of rats receiving their fluorine supplements in the drinking water were generally very well developed by the beginning of the eighth week, there being no distinction between the rats getting  $\text{NaF}$  and those getting  $\text{CaF}_2$ . The trio-mates receiving  $\text{NaF}$  in the food showed only incipient dental striations at this time.

The average growth data and the average results of the carcass analyses are assembled in table 1. There were no significant distinctions in any item listed between rats 1 and 2 of the ten trios, receiving  $\text{NaF}$  and  $\text{CaF}_2$  in the drinking water. Evidently fluorine administered in these two forms at low levels of consumption is equally assimilable and presumably equally toxic. However, between the first two rats and the third rat in the various trios, clear distinctions are apparent, indicating a smaller toxicity of fluorine when consumed in food than when consumed in water. The most significant comparisons are those between rats receiving  $\text{NaF}$  in their drinking water and those receiving the same compound in the same amounts in their food. The statistical analyses of such comparisons are recorded in table 2. With reference to the fluorine content of bones, teeth and soft tissues and to the total fluorine retention, the rats receiving  $\text{NaF}$  in their water definitely surpassed those receiving  $\text{NaF}$  in their food. Stated in another way, the assimilation of fluorine was depressed by admixture with food 19.3% in the bone, 14.0% in the teeth, 29.0% in the soft tissues, and 21.5% in the entire carcass. The latter figure compares well with that obtained previously in a similar experiment comparing the assimilation of fluorine consumed as cryolite in water and in food (Lawrenz, Mitchell and Ruth, '39 b). In this case an average depression of 20.4% was observed due to admixture with food. The failure of McClure ('39) to detect any such difference may probably be

TABLE I  
*The growth data and the fluorine metabolism data secured in the two experiments. Averages for ten trios in experiment 1 and for twelve trios in experiment 2.*

	EXPERIMENT 1. AVERAGES FOR RATS RECEIVING			EXPERIMENT 2. AVERAGES FOR RATS RECEIVING		
	Calcium fluoride in water	Sodium fluoride in water	Sodium fluoride in food	Sodium fluoride	Green tea	Rook phosphate
Initial body weight, gm.	48	48	48	39	39	40
Total gain in weight, gm.	149	151	150	176	166	177
Final body length, mm.	210	213	206	214	214	216
Total food consumed, gm.	648	648	648	750	750	750
Number of food refusals	15	13	11	12	5	12
Total intake of fluorine, mg.	6.56	6.56	6.56	8.80	8.80	8.80
Feeding period, days	98	98	98	86	86	86
Final empty body weight, gm.	197	199	198	215	205	217
Weight of dry, fat-free bones, gm.	10.136	10.289	10.170	10.628	10.516	10.665
Weight of dry teeth, gm.	0.426	0.428	0.432	0.398	0.405	0.396
Weight of soft tissues, gm.	179	182	179	194	184	196
Fluorine in bones, p.p.m.	298	290	238	284	272	258
Fluorine in bones, mg.	2.986	2.951	2.380	3.010	2.847	2.743
Fluorine in teeth, p.p.m.	198	196	167	230	209	199
Fluorine in teeth, mg.	0.084	0.084	0.072	0.091	0.085	0.079
Fluorine in soft tissues, p.p.m.	0.42	0.44	0.32	0.26	0.26	0.26
Fluorine in soft tissues, mg.	0.076	0.081	0.057	0.050	0.048	0.050
Total fluorine in carcass, mg.	3.146	3.116	2.509	3.151	2.980	2.872
Initial fluorine content, mg.	0.312	0.314	0.311	0.188	0.187	0.190
Total fluorine retained, mg.	2.834	2.802	2.198	2.963	2.793	2.682
Total fluorine retained, per cent	48.2	42.9	33.6	35.7	31.7	30.5

TABLE 2  
Statistical analysis of fluorine metabolism data.

FLUORINE	EXPERIMENT 1. SODIUM FLUORIDE IN WATER VERSUS SODIUM FLUORIDE IN FOOD				EXPERIMENT 2. SODIUM FLUORIDE VERSUS GREEN TEA			
	Mean difference <sup>1</sup>	Standard deviation of differences	Mean difference <sup>3</sup>	Standard deviation <sup>3</sup> of differences	Mean difference <sup>1</sup>	Standard deviation of differences	t value	P <sup>2</sup>
In bones, p.p.m.	52.6	31.6	4.99	0.0004	12.2	18.9	2.16	0.027
In bones, mg.	0.570	0.209	8.20	0.0004	0.163	0.256	2.11	0.029
In teeth, p.p.m.	28.7	15.3	5.63	0.0002	20.2	25.0	2.69	0.010
In teeth, mg.	0.0117	0.0071	4.97	0.0004	0.0066	0.0085	2.57	0.013
In soft tissues, p.p.m.	0.128	0.056	6.85	0.0004	Insignificant	Insignificant	Insignificant	Insignificant
In soft tissues, mg.	0.0235	0.0106	6.65	0.0004	Insignificant	Insignificant	Insignificant	Insignificant
Total retained, mg.	0.603	0.218	8.31	0.0005	0.170	0.257	2.19	0.025

<sup>1</sup>The positive sign indicates that the mean difference favors the sodium fluoride administered in water.

<sup>2</sup>The probability according to Student ('25) that a mean difference as large or larger than that observed, and in the same direction, would result from the fortuitous combination of the many uncontrolled factors undoubtedly operating.

<sup>3</sup>A positive sign indicates that the mean difference is in favor of the sodium fluoride.

<sup>4</sup>A positive sign indicates that the mean difference is in favor of the green tea.

ascribed to the fact that in his experiments no attempt was made to separate in time the consumption of water and of food by the experimental rats, thus permitting the obscuration of the very effect it was desired to measure.

*Experiment 2.* The rats in this experiment grew somewhat better than those in experiment 1, making gains averaging about 2.0 gm. daily, except for the rats on the green tea ration, which consistently grew at a somewhat slower rate than those on the other two rations. Striations appeared on the incisor teeth of the rats generally at the beginning of the seventh week of feeding, and generally were more completely developed in the rat receiving the diet containing sodium fluoride. With two of the rats, one receiving sodium fluoride and one receiving rock phosphate, this depigmentation of the enamel of the teeth proceeded to completion.

The average results of the experiment are summarized in table 1, and the statistical analyses of differences observed between the sodium fluoride diet and the tea diet, and between the latter and the rock phosphate diet are presented in table 2. On the average, the assimilation of fluorine was most complete from the sodium fluoride diet, least complete from the rock phosphate diet, and intermediate from the green tea diet. The differences between fluorine accumulations in bones and teeth promoted by the sodium fluoride diet and the tea diet were statistically significant, taking a value of  $P$  of 0.03 or less as being negligible. The total retention of fluorine also was significantly different in this comparison, being 5.4% less on the tea diet. The soft tissues secured on the two diets revealed no difference in fluorine content that could be attributed to a difference in the source of dietary fluorine. With this one exception, the fluorine of sodium fluoride was also distinctly and significantly more assimilable than the fluorine of rock phosphate, the average difference in total fluorine retention being 9.0% in this case.

A comparison of the results secured on the tea diet with those secured on the rock phosphate diet, reveals a greater

average assimilation of the fluorine of tea in the bones, teeth and total carcass, but the differences are borderline in significance.

#### SUMMARY AND CONCLUSIONS

Comparisons have been made by controlled feeding experiments on growing rats, involving twenty-two trio groups, of the retention of dietary fluorine from supplements of sodium fluoride, calcium fluoride, green tea and raw rock phosphate, and from sodium fluoride administered in water and in food. In the latter comparison, the consumptions of water and of food were separated in time as much as possible in order to measure the unobscured effect. In the comparison of sodium fluoride and calcium fluoride, the salts were dissolved in water. In all experiments the fluorine was administered at levels sufficiently low (9 to 12 p.p.m.) to be of significance with reference to the fluorine hazard in human nutrition. The data secured support the following conclusions:

1. At these low levels of intake, the fluorine in sodium fluoride is no more assimilable by the animal body, and presumably no more toxic, than the fluorine in calcium fluoride. It is, however, definitely more assimilable (5%) than the fluorine in green tea, which in turn is probably somewhat more assimilable than the fluorine in raw rock phosphate.

2. The fluorine of sodium fluoride, administered in the drinking water at low levels, is 21% more completely assimilated by the animal body than the fluorine of the same compound consumed in the same amounts in the food. In a similar experiment previously carried out with cryolite as the source of fluorine, the depression in assimilation brought about by admixture with food was 20%.

#### LITERATURE CITED

- COULSON, E. J., R. E. REMINGTON AND K. M. LYNCH 1935 Metabolism in the rat of the naturally occurring arsenic of shrimp as compared with arsenic trioxide. *J. Nutrition*, vol. 10, pp. 255-270.  
ELLIS, G., AND L. A. MAYNARD 1936 Effect of low levels of fluorine intake on bones and teeth. *Proc. Soc. Exp. Biol. Med.*, vol. 35, pp. 12-16.

- EVANS, R. J., AND P. H. PHILLIPS 1939 A study of the comparative toxicity of cryolite fluorine and sodium fluoride for the rat. *Am. J. Physiol.*, vol. 126, pp. 713-719.
- LAWRENZ, M., AND H. H. MITCHELL 1941 The effect of dietary calcium and phosphorus on the assimilation of dietary fluorine. *J. Nutrition*, vol. 22, pp. 91-101.
- LAWRENZ, M., H. H. MITCHELL AND W. A. RUTH 1939 a The comparative toxicity of fluorine in calcium fluoride and in cryolite. *J. Nutrition*, vol. 18, pp. 115-125.
- 1939 b A comparison of the toxicity of fluorine in the form of cryolite administered in water and in food. *J. Nutrition*, vol. 18, pp. 127-141.
- 1940 a The comparative assimilation of fluorine by growing rats during continuous and intermittent dosage. *J. Nutrition*, vol. 20, pp. 383-390.
- 1940 b Adaptation of the growing rat to the ingestion of a constant concentration of fluorine in the diet. *J. Nutrition*, vol. 19, pp. 531-546.
- LEE, C. F., AND H. W. NILSON 1939 Study of the metabolism of naturally occurring fluorine in canned salmon and mackerel. U. S. Dept. Com., Bur. Fisheries, Invest. Rpt. No. 44.
- MCCLURE, F. J. 1939 Fluorides in food and drinking water. A comparison of effects of water-ingested versus food ingested sodium fluoride. *Natl. Inst. of Health, Bul.* No. 172, 53 pp.
- REID, E. 1936 The fluorine content of some Chinese food materials. *Chinese J. Physiol.*, vol. 10, pp. 259-271.
- SMITH, M. C., AND R. M. LEVERTON 1934 Comparative toxicity of fluorine compounds. *Ind. Eng. Chem.*, vol. 26, pp. 791-797.
- STUDENT 1925 New tables for testing the significance of observations. *Metron*, vol. 5, pp. 105-120.



# INDEX

- A**BSORPTION, and digestion, of the carbohydrate of a complete diet, effects of various vitamin supplements and of whole yeast on . . . . .  
 Achromotrichia, nutritional, in rats studies on . . . . .  
 Achromotrichia, nutritional, of rats, inefficacy of hormones in . . . . .  
 Acid, ascorbic, content of cow's milk during pregnancy . . . . .  
 Acid, nicotinic, and coenzyme content of the tissue of normal and black-tongue dogs . . . . .  
 Acid, nicotinic, content of meat and meat products . . . . .  
 Acid, nicotinic, riboflavin, pantothenic acid and thiamine in rye and its milled products . . . . .  
 Acids, unsaturated fatty, and fats, effect of certain, upon utilization of carotene . . . . .  
 Adaptometer, dark, and blood vitamin A measurements in a North Carolina nutrition survey . . . . .  
 Albino rat, growth curve of, in relation to diet . . . . .  
 Amblystoma tigrinum, larval, dietary production of cataracts in . . . . .  
 ANDERSON, HARLAN D., AND ALVIN L. MOXON. The excretion of selenium by rats on a seleniferous wheat ration . . . . .  
 Apparent digestibility of carbohydrates, fats, and "indigestible residue" in whole wheat and white breads . . . . .  
 Ascorbic acid content of cow's milk during pregnancy . . . . .  
 Assay method, rat growth, for riboflavin, studies on . . . . .  
 Assimilation of dietary fluorine, effect of dietary calcium and phosphorus on . . . . .  
 Assimilation of fluorine by rats from natural and synthetic cryolite and from cryolite-sprayed fruits . . . . .  
 Assimilation, relative, of fluorine from fluorine-bearing minerals and food (tea), and from water and food . . . . .  
 Autoclaving, effect of, on nutritive value of proteins in cottonseed meal . . . . .  
 Availability of the iron of beef muscle, effect of heat on . . . . .  
 Avian thiamine deficiency. Characteristic symptoms and their pathogenesis. III . . . . .  
  
**B**ALANCE studies, magnesium, with infants . . . . .  
 Balanced rations composed of combinations of different feeds, utilization by calves of energy contained in . . . . .  
 BAROTT, HERBERT G., AND EMMA M. PRINGLE. Energy and gaseous metabolism of the hen as affected by temperature . . . . .  
  
 287 Basal metabolism and endogenous nitrogen metabolism subsequently determined, relationship between, influence of plane of nutrition and of environmental temperature on . . . . .  
 553 BASINSKI, DANIEL H. See Sealock Robert R. . . . .  
 565 Beef muscle, iron of, effect of heat on availability of . . . . .  
 267 Behavior of rats and mice towards deficiencies of certain members of the vitamin B complex, differences in . . . . .  
 409 BESSEY, OTTO A. See Swank, R. L. . . . .  
 535 BIELY, JACOB. See Billings, F. L. . . . .  
 BILLINGS, F. L., JACOB BIELY, HERBERT FISHER AND CARL HEDREN. The riboflavin content of fish products . . . . .  
 527 Biological value, and digestibility, of whole wheat breads as compared with white bread . . . . .  
 153 Blacktongue and normal dogs, tissues of, nicotinic acid and coenzyme content of . . . . .  
 597 409 Blood plasma, magnesium content of, effect of magnesium salts and various natural feeds upon. Magnesium studies in calves. II . . . . .  
 365 609 Blood vitamin A measurements, and dark adaptometer, in a North Carolina nutrition survey . . . . .  
 103 BODANSKY, MEYER, AND VIRGINIA B. DUFF. Dependence of fetal growth and storage of calcium and phosphorus on the parathyroid function and diet of pregnant rats . . . . .  
 589 25 Bread, white, as compared with whole wheat breads, digestibility and biological value of . . . . .  
 267 573 Breads, whole wheat and white, apparent digestibility of carbohydrates, fats, and "indigestible residue" in . . . . .  
 399 589 Broccoli and cauliflower, utilization of calcium of . . . . .  
 91 477  
 451  
  
 621 **C**ALCIFICATION and growth on a diet deficient in phosphate but otherwise adequate . . . . .  
 481 Calcium and phosphorus, effect of dietary, on assimilation of dietary fluorine . . . . .  
 197 91 Calcium and phosphorus, storage of, and fetal growth, dependence of, on the parathyroid function and diet of pregnant rats . . . . .  
 77 25 Calcium deficiency (severe) in growing rats. III. Serum calcium of individual animals during development of calcium deficiency . . . . .  
 53 1 Calcium levels (different), influence of protein intake upon growth, reproduction and longevity studied at . . . . .  
 541 827 Calcium of cauliflower and broccoli, utilization of . . . . .  
 273 477

- Calcium, serum, of individual animals during development of calcium deficiency. Severe calcium deficiency in growing rats. III  
Calves, magnesium studies in. II. The effect of magnesium salts and various natural feeds upon the magnesium content of the blood plasma  
Calves, utilization by, of energy contained in balanced rations composed of combinations of different feeds  
Carbohydrate of a complete diet, effects of various vitamin supplements and of whole yeast on digestion and absorption of  
Carbohydrate storage and mobilization in rat  
Carbohydrates, fats, and "indigestible residue" in whole wheat and white breads, apparent digestibility of  
Caries, experimental rat, reduction in, by fluorine  
Caries, induced dental, in rats, observations on. III. Effect of fluoride on rat caries and on composition of rats' teeth  
Carotene, effect of certain fats and unsaturated fatty acids upon utilization of  
Casein diets, lactation-promoting properties of cystine when added to. Dietary requirements for fertility and lactation. XXVIII  
Cataracts in larval *Ambystoma tigrinum*, dietary production of  
Cauliflower and broccoli, utilization of calcium of  
CEREcedo, LEOPOLD R. See Foy, John R.  
Characteristic symptoms and their pathogenesis. III. Avian thiamine deficiency  
Child and mother, influence of prenatal diet on  
Choline deficiency in young rats, improved diets for nutritional and pathologic studies of  
Choline, nutritional importance of. Editorial review  
Coenzyme and nicotinic acid content of the tissues of normal and black-tongue dogs  
Complex, vitamin B, and fat metabolism  
Complex, vitamin B, deficiencies of certain members of, differences in behavior of rats and mice towards  
Composition of rats' teeth, effect of fluoride on rat caries and on. Observations on induced dental caries in rats  
Compounds, certain organic, and other dietary supplements, effect of, on perosis  
CONLEY, C. L. See Huffman, C. F.  
CONNER, ROBERT T. See Kao, Hsueh-Chung  
Content, ascorbic acid, of cow's milk during pregnancy  
Content, coenzyme and nicotinic acid, of the tissues of normal and black-tongue dogs  
Content, magnesium, of blood plasma, effect of magnesium salts and various natural feeds upon. Magnesium studies in calves. II  
Content, nicotinic acid, of meat and meat products .....
- Content, riboflavin, of fish products .. 425  
Content, vitamin, of eggs, effect of incubation on .. 483  
Cottonseed meal, proteins in, effect of autoclaving on nutritive value of .. 431  
Cottonseed oil, hydrogenated, and steam-rendered lard, nutritive properties of .. 65  
609 CowGILL, GEORGE R. See Street, Harold R. .... 7  
Cow's milk during pregnancy, ascorbic acid content of .. 267  
541 Cryolite, natural and synthetic, and cryolite-sprayed fruits, assimilation of fluorine by rats from .. 451  
287 Curve, growth, of the albino rat in relation to diet .. 123  
205 Cystine, lactation-promoting properties of, when added to casein diets. Dietary requirements for fertility and lactation. XXVIII .. 491  
589 DANN, WILLIAM J., AND PHILIP 255 HANDLEY. The nicotinic acid and coenzyme content of the tissues of normal and blacktongue dogs .. 409  
DANN, W. J. See Yarbrough, M. E. 597  
391 Dark adaptometer and blood vitamin A measurements in a North Carolina nutrition survey .. 597  
153 DAY, HARRY G. See Folis, Richard H., Jr. .... 223  
Deficiencies of certain members of the vitamin B complex, differences in behavior of rats and mice towards .. 439  
365 Deficiency, avian thiamine. Characteristic symptoms and their pathogenesis. III .. 77  
477 Deficiency, choline, in young rats, improved diets for nutritional and pathologic studies of .. 109  
439 Deficiency, riboflavin, in dog, further observations of .. 7  
515 Deficiency, riboflavin, in rat, pathology of .. 345  
Deficiency, severe calcium, in growing rats. III. Serum calcium of individual animals during development of calcium deficiency .. 1  
289 Dental caries (induced) in rats, observations on. III. Effect of fluoride on rat caries and on composition of rats' teeth .. 391  
409 Dependence of fetal growth and storage of calcium and phosphorus on the parathyroid function and diet of pregnant rats .. 25  
439 DEUEL, HARRY J., JR., NELLIE HALLDAY, LOIS FIELD HALLMAN, CORNELIA JOHNSTON AND ALBERT J. MILLER. The production of high vitamin A milk by diet .. 303  
391 Diet and parathyroid function of pregnant rats, dependences of fetal growth and storage of calcium and phosphorus on .. 25  
315 Diet (complete), carbohydrate of, effects of various vitamin supplements and of whole yeast on digestion and absorption of .. 287  
609 Diet deficient in phosphate but otherwise adequate, growth and calcification on .. 139  
409 Diet extremely low in zinc, histological studies of tissues of rats fed .. 223  
609 Diet, growth curve of albino rat in relation to .. 123  
585 Diet, prenatal, influence of, on mother and child .. 515

- Diet, production of high vitamin A milk by ..... 303  
 Dietary calcium and phosphorus, effect of, on assimilation of dietary fluorine ..... 91  
 Dietary production of cataracts in larval *Amblystoma tigrinum* ..... 365  
 Dietary requirements for fertility and lactation. XXVIII. The lactation-promoting properties of cystine when added to casein diets ..... 491  
 Dietary requirements for fertility and lactation. XXIX. The existence of a new dietary factor essential for lactation ..... 499  
 Dietary supplements (other) and certain organic compounds, effect of, on perosis ..... 315  
 Diets, casein, lactation-promoting properties of cystine when added to. Dietary requirements for fertility and lactation. XXVIII ..... 491  
 Diets (improved) for nutritional and pathologic studies of choline deficiency in young rats ..... 109  
 Differences in behavior of rats and mice towards deficiencies of certain members of the vitamin B complex ..... 439  
 Digestibility and biological value of whole wheat breads as compared with white bread ..... 573  
 Digestibility, apparent, of carbohydrates, fats, and "indigestible residue" in whole wheat and white breads ..... 589  
 Digestion and absorption of the carbohydrate of a complete diet, effects of various vitamin supplements and of whole yeast on dl-a-tocopherol, minimum requirement of rabbits for ..... 287  
 Dog, further observations of riboflavin deficiency in ..... 415  
 Dogs, tissues of normal and black-tongue, nicotinic acid and coenzyme content of ..... 7  
 DUFF, VIRGINIA B. See Bodansky, Meyer ..... 409  
 DUNCAN, C. W. See Huffman, C. F.  
 Dystrophy, muscular, in the young, prevention of, and reproduction in the female, a-tocopherol requirement of the rat for ..... 25  
 EBBBS, JOHN H., FREDERICK F. TISDALE AND WILLIAM A. SCOTT. The influence of prenatal diet on the mother and child ..... 609  
 Economy of food utilization, further observations on riboflavin as a food factor in ..... 515  
 Editorial review. The nutritional importance of choline ..... 481  
 Effect of autoclaving on the nutritive value of proteins in cottonseed meal ..... 295  
 Effect of certain fats and unsaturated fatty acids upon utilization of carotene ..... 239  
 Effect of certain organic compounds and other dietary supplements on perosis ..... 431  
 Effect of dietary calcium and phosphorus on the assimilation of dietary fluorine ..... 315  
 Effect of fluoride on rat caries and on composition of rats' teeth. Observations on induced dental caries in rats. III ..... 91  
 Effect of heat on availability of the iron of beef muscle ..... 197  
 Effect of incubation on vitamin content of eggs ..... 483  
 Effect of magnesium salts and various natural feeds upon magnesium content of blood plasma. Magnesium studies in calves. II ..... 609  
 Effects of various vitamin supplements and of whole yeast on digestion and absorption of the carbohydrate of a complete diet ..... 287  
 Eggs, effect of incubation on vitamin content of ..... 483  
 ELVEHJEM, C. A. See McIntire, J. M.  
 Endogenous nitrogen metabolism and basal metabolism subsequently determined, relationship between, influence of plane of nutrition and of environmental temperature on ..... 333  
 Endogenous nitrogen metabolism of rats, influence of previous regimes of protein feeding on ..... 383  
 Energy and gaseous metabolism of the hen as affected by temperature ..... 273  
 Energy contained in balanced rations composed of combinations of different feeds, utilization by calves of ..... 541  
 ENGEL, RUBEN W., AND WILLIAM D. SALMON. Improved diets for nutritional and pathologic studies of choline deficiency in young rats ..... 109  
 Environmental temperature and plane of nutrition, influence of, on relationship between basal metabolism and endogenous nitrogen metabolism subsequently determined ..... 333  
 EPSTEIN, SAMUEL H., AND SERGIUS MORGULIS. The minimum requirement of rabbits for dl-tocopherol ..... 415  
 Excretion of selenium by rats on a seleniferous wheat ration ..... 103  
 Existence of a new dietary factor essential for lactation. Dietary requirements for fertility and lactation. XXIX ..... 499  
 Experimental rat caries, reduction in, by fluorine ..... 255  
 FACTOR, food, in economy of food utilization, further observations on riboflavin as ..... 295  
 Factor, new dietary, essential for lactation, existence of. Dietary requirements for fertility and lactation. XXIX ..... 499  
 Fat metabolism, and vitamin B complex ..... 359  
 Fats and unsaturated fatty acids, effect of certain, upon utilization of carotene ..... 153  
 Fats, carbohydrates, and "indigestible residue" in whole wheat and white breads, apparent digestibility of ..... 559  
 Feeds (combinations of different), utilization by calves of energy contained in balanced rations composed of ..... 589  
 Feeds (various natural) and magnesium salts, effect of, upon magnesium content of blood plasma. Magnesium studies in calves. II ..... 541  
 Female, reproduction in, and prevention of muscular dystrophy in the young, a-tocopherol requirement of rat for ..... 609  
 Fertility and lactation, dietary requirements for ..... 463  
 Fertility and lactation, dietary requirements for ..... 491, 499

- |   |   |
|---|---|
| Fetal growth and storage of calcium and phosphorus, dependence of, on parathyroid function and diet of pregnant rats  | Growing rats, severe calcium deficiency in  |
| FINCKE, MARGARET L. The utilization of the calcium of cauliflower and broccoli  | III. Serum calcium of individual animals during development of calcium deficiency   |
| FINN, SIDNEY B., AND HAROLD C. HODGE. Reduction in experimental rat caries by fluorine  | Growth and calcification on a diet deficient in phosphate but otherwise adequate  |
| Fish products, riboflavin content of  | Growth assay method, rat, for riboflavin, studies on  |
| FISHER, HERBERT. See Billings, F. L.  | Growth curve of the albino rat in relation to diet  |
| Fluoride, effect of, on rat caries and on composition of rats' teeth. Observations on induced dental caries in rats. III  | Growth, fetal, and storage of calcium and phosphorus, dependence of, on parathyroid function and diet of pregnant rats  |
| Fluorine, assimilation of, by rats from natural and synthetic cryolite and from cryolite-sprayed fruits   | Growth, reproduction and longevity studied at different calcium levels, influence of protein intake upon  |
| Fluorine, dietary, effect of dietary calcium and phosphorus on assimilation of  | GUEST, MASON M. Carbohydrate storage and mobilization in the rat  |
| Fluorine, reduction in experimental rat caries by   | GUSTAVSON, R. G. See Wilgus, Herbert S., Jr.  |
| Fluorine, relative assimilation of, from fluorine-bearing minerals and food (tea), and from water and food  | HAINES, W. T. See Mitchell, H. H.   |
| FOLLIS, RICHARD H., JR., HARRY G. DAY AND E. V. McCULLUM. Histological studies of the tissues of rats fed a diet extremely low in zinc  | HALL, LILLIAN. See Zucker, Theodore F.  |
| FONTAINE, THOMAS D. See Olcott, Harold S.   | HALLIDAY, NELLIE. See Deuel, Harry J., Jr.  |
| Food and water, and fluorine-bearing minerals and food (tea), relative assimilation of fluorine from  | HALLMAN, LOIS F. See Deuel, Harry J., Jr.   |
| Food factor in economy of food utilization, further observations on riboflavin as   | HAMILTON, T. S. See Mitchell, H. H.   |
| FORBES, JOHN C. Vitamin B complex and fat metabolism  | HANDLER, PHILIP. See Dunn, William J.   |
| FOY, JOHN R., AND LEOPOLD R. CERECCEDO. Differences in the behavior of rats and mice towards deficiencies of certain members of the vitamin B complex   | HARRIS, LOREN E., AND HAROLD H. MITCHELL. The value of urea in the synthesis of protein in the paunch of the ruminant. I. In maintenance  |
| FRENCH, ROWLAND B., JOSEPH I. ROUTE AND HENRY A. MATTILL. The influence of previous regimes of protein feeding on the endogenous nitrogen metabolism of rats  | HARRIS, LOREN E., AND HAROLD H. MITCHELL. The value of urea in the synthesis of protein in the paunch of the ruminant. II. In growth  |
| Fruits, cryolite-sprayed, and natural and synthetic cryolite, assimilation of fluorine by rats from   | Heat, effect of, on availability of the iron of beef muscle   |
| Function, parathyroid, and diet of pregnant rats, dependence of fetal growth and storage of calcium and phosphorus on   | HEDREEN, CARL. See Billings, F. L.  |
| GASEOUS and energy metabolism of hen as affected by temperature   | Hen, energy and gaseous metabolism of, as affected by temperature   |
| GASSNER, F. X. See Wilgus, Herbert S., Jr.  | HENDERSON, LAVELL M. See McIntire, J. M.  |
| GOETTSCH, MARIANNE, AND ALWIN M. PAPPENHEIMER. $\alpha$ -tocopherol requirement of the rat for reproduction in the female and prevention of muscular dystrophy in the young                             | High vitamin A milk by diet, production of  |
| Goitrogenicity of soybeans  | Histological studies of the tissues of rats fed a diet extremely low in zinc  |
| GREENBERG, DAVID M., AND WILFRED D. MILLER. Severe calcium deficiency in growing rats. III. Serum calcium of individual animals during development of calcium deficiency                                | HOAGLAND, RALPH, AND GEORGE G. SNIDER. Nutritive properties of steam-rendered lard and hydrogenated cottonseed oil  |
| GRIFFITH, WENDELL H. Editorial review. The nutritional importance of choline  | HODGE, HAROLD C. See Finn, Sidney B.  |
| HUFFMAN, O. F., C. L. CONLEY, C. C. LIGHTFOOT AND C. W. DUNCAN. Magnesium studies in calves. II. The effect of magnesium salts and various natural feeds upon the magnesium content of the blood plasma | HOLMES, ARTHUR D., FRANCIS TRIPP, ELMER A. WOELFFER AND GEORGE H. SATTERFIELD. The ascorbic acid content of cow's milk during pregnancy   |
| Hydrogenated cottonseed oil and steam-rendered lard, nutritive properties of  | Hormones, inefficacy of, in nutritional achromotrichia of rats  |
| 1   | HUFFMAN, O. F., C. L. CONLEY, C. C. LIGHTFOOT AND C. W. DUNCAN. Magnesium studies in calves. II. The effect of magnesium salts and various natural feeds upon the magnesium content of the blood plasma |
| 239   | Hydrogenated cottonseed oil and steam-rendered lard, nutritive properties of  |

- I** HDE, AARON J., AND HENRY A. SCHUETTE. Thiamine, nicotinic acid, riboflavin, and pantothenic acid in rye and its milled products Improved diets for nutritional and pathologic studies of choline deficiency in young rats . . . . .
- Incubation, effect of, on vitamin content of eggs . . . . .
- "Indigestible residue" in whole wheat and white breads, apparent digestibility of carbohydrates, fats, and Inefficacy of hormones in nutritional achromotrichia of rats . . . . .
- Infants, magnesium balance studies with . . . . .
- Iron of beef muscle, effect of heat on availability of . . . . .
- J** JOHNSTON, CORNELIA. See Deuel, Harry J., Jr. . . . .
- JUKES, THOMAS H. The effect of certain organic compounds and other dietary supplements on perosis . . . . .
- K** KAO, HSUEH-CHUNG, ROBERT T. CONNER AND HENRY C. SHERMAN. Influence of protein intake upon growth, reproduction and longevity studied at different calcium levels . . . . .
- KNOTT, ELIZABETH M. See Shukers, Carroll F. . . . .
- KOCHAKIAN, CHARLES D. See Murlin, John R. . . . .
- L** ACTATION, existence of a new dietary factor essential for. Dietary requirements for fertility and lactation. XXIX . . . . .
- Lactation-promoting properties of cystine when added to casein diets. Dietary requirements for fertility and lactation. XXVIII . . . . .
- Lard, steam-rendered, and hydrogenated cottonseed oil, nutritive properties of . . . . .
- Larval *Amblystoma tigrinum*, dietary production of cataracts in . . . . .
- LAWRENZ, MARGARET, AND HAROLD H. MITCHELL. The effect of dietary calcium and phosphorus on the assimilation of dietary fluorine . . . . .
- LAWRENZ, MARGARET, AND HAROLD H. MITCHELL. The assimilation of fluorine by rats from natural and synthetic cryolite and from cryolite-sprayed fruits . . . . .
- LAWRENZ, MARGARET, AND HAROLD H. MITCHELL. The relative assimilation of fluorine from fluorine-bearing minerals and food (tea), and from water and food . . . . .
- Levels, different calcium, influence of protein intake upon growth, reproduction and longevity studied at . . . . .
- LIGHTFOOT, C. C. See Huffman, C. F. Longevity, growth and reproduction studied at different calcium levels, influence of protein intake upon . . . . .
- M** McCCLURE, FRANK J. Observations on induced dental caries in rats. III. Effect of fluorine on rat caries and on composition of rats' teeth . . . . .
- McCOLLUM, E. V. See Folllis, Richard H., Jr. . . . . .
- MCINTIRE, JUNIUS M., HARRY A. WAISMAN, LAVELL M. HENDERSON AND CONRAD A. ELVERHJEM. Nicotinic acid content of meat and meat products . . . . .
- 527 Magnesium balance studies with infants . . . . .
- 109 Magnesium studies in calves. II. The effect of magnesium salts and various natural feeds upon the magnesium content of the blood plasma . . . . .
- 483 MARSHALL, MARGARET E. See Murlin, John R. . . . .
- 565 MATTILL, HENRY A. See French, Rowland B. . . . .
- 589 Meal, cottonseed, proteins in, effect of autoclaving on nutritive value of . . . . .
- 197 Meat and meat products, nicotinic acid content of . . . . .
- 303 Metabolism, basal, and endogenous nitrogen metabolism subsequently determined, relationship between, influence of plane of nutrition and of environmental temperature on . . . . .
- 315 Metabolism, endogenous nitrogen, of rats, influence of previous regimes of protein feeding on . . . . .
- 327 Metabolism, energy and gaseous, of hen as affected by temperature . . . . .
- 53 Metabolism, fat, and vitamin B complex . . . . .
- 573 Method, rat growth assay, for riboflavin, studied on . . . . .
- 58 Mice and rats, differences in behavior of, towards deficiencies of certain members of vitamin B complex . . . . .
- 65 Milk, cow's, during pregnancy, ascorbic acid content of . . . . .
- 365 Milk, high vitamin A, by diet, production of . . . . .
- 499 Milled products, rye and its, thiamine, nicotinic acid, riboflavin and pantothenic acid in . . . . .
- 491 MILLER, ALBERT J. See Deuel, Harry J., Jr. . . . .
- 65 MILLER, WILBUR D. See Greenberg, David M. . . . .
- 365 Minerals, fluorine-bearing, and food (tea), and water and food, relative assimilation of fluorine from . . . . .
- 491 Minimum requirement of rabbits for dl-a-tocopherol . . . . .
- 91 MITCHELL, H. H. AND T. S. HAMILTON, WITH TECHNICAL ASSISTANCE OF W. T. HAINES. The utilization by calves of the energy contained in balanced rations composed of combinations of different feeds . . . . .
- 451 MITCHELL, HAROLD H. See Harris, Lorin E. . . . . .
- 621 MITCHELL, HAROLD H. See Lawrenz, Margaret . . . . . .
- 327 MITCHELL, HAROLD H. See Treichler, Ray . . . . .
- 621 Mobilization and carbohydrate storage in rat . . . . .
- 609 MORGULIS, SERGIUS. See Epstein, Samuel H. . . . .
- 327 Mother and child, influence of prenatal diet on . . . . .
- MOXON, ALVIN L. See Anderson, Harlan D. . . . . .
- 391 MURLIN, JOHN R., MARGARET E. MARSHALL AND CHARLES D. KOCHAKIAN. Digestibility and biological value of whole wheat breads as compared with white bread . . . . . .

- MURLIN, JOHN R. See Sealock, Robert R. . . . .
- Muscle, beef, iron of, effect of heat on availability of . . . . .
- Muscular dystrophy in the young, prevention of, and reproduction in the female,  $\alpha$ -tocopherol requirement of the rat for . . . . .
- MUSSETT, CHARLES W., AND KLAUS UNNA. Inefficacy of hormones in nutritional achromotrichia of rats . . . . .
- NASSET, EDMUND S. See Russell, Raymond A. . . . .
- Natural and synthetic cryolite and cryolite-sprayed fruits, assimilation of fluorine by rats from . . . . .
- Natural feeds (various), and magnesium salts, effect of, upon magnesium content of blood plasma. Magnesium studies in calves. II
- Nicotinic acid and coenzyme content of the tissue of normal and black-tongue dogs . . . . .
- Nicotinic acid content of meat and meat products . . . . .
- Nicotinic acid, riboflavin, pantothenic acid and thiamine in rye and its milled products . . . . .
- Nitrogen metabolism, endogenous, and basal metabolism subsequently determined, relationship between, influence of plane of nutrition and of environmental temperature on
- Nitrogen metabolism, endogenous, of rats, influence of previous regimes of protein feeding on . . . . .
- Normal and blacktongue dogs, tissues of, nicotinic acid and coenzyme content of . . . . .
- North Carolina nutrition survey, dark adaptometer and blood vitamin A measurements in . . . . .
- Nutrition, plane of, and environmental temperature, influence of, on relationship between basal metabolism and endogenous nitrogen metabolism subsequently determined . . . . .
- Nutrition survey (North Carolina), dark adaptometer and blood vitamin A measurements in . . . . .
- Nutritional achromotrichia in rats, studies on . . . . .
- Nutritional achromotrichia of rats, inefficacy of hormones in . . . . .
- Nutritional and pathologic studies of choline deficiency in young rats, improved diets for . . . . .
- Nutritional importance of choline. Editorial review . . . . .
- Nutritive properties of steam-rendered lard and hydrogenated cottonseed oil . . . . .
- Nutritive value of proteins in cottonseed meal, effect of autoclaving on . . . . .
- OBSERVATIONS (further) of riboflavin deficiency in the dog . . . . .
- Observations (further) on riboflavin as a food factor in economy of food utilization . . . . .
- Observations on induced dental caries in rats. III. Effect of fluoride on rat caries and on composition of rats' teeth . . . . .
- Oil, hydrogenated cottonseed, and steam-rendered lard, nutritive properties of . . . . .
- OLCOTT, HAROLD S., AND THOMAS D. FONTAINE. The effect of autoclaving on the nutritive value of the proteins in cottonseed meal . . . . .
- OLDHAM, HELEN G. The effect of heat on the availability of the iron of beef muscle . . . . .
- Organic compounds (certain), and other dietary supplements, effect of, on perosis . . . . .
- PANTOTHENIC acid, thiamine, nicotinic acid and riboflavin in rye and its milled products . . . . .
- PAPPENHEIMER, ALWIN M. See Goetsch, Marianne . . . . .
- Parathyroid function and diet of pregnant rats, dependence of fetal growth and storage of calcium and phosphorus on . . . . .
- PATCH, ESTHER M. Dietary production of cataracts in larval *Ambystoma tigrinum* . . . . .
- Pathogenesis (their), characteristic symptoms and. III. Avian thiamine deficiency . . . . .
- Pathologic and nutritional studies of choline deficiency in young rats, improved diets for . . . . .
- Pathology of riboflavin deficiency in rat . . . . .
- PATTON, ALVA R. See Wilgus, Herbert S., Jr. . . . .
- Paunch of ruminant, value of urea in synthesis of protein in. I. In maintenance . . . . .
- II. In growth . . . . .
- Perosis, effect of certain organic compounds and other dietary supplements on . . . . .
- PHILLIPS, PAUL H. See Shaw, James H. . . . .
- Phosphate, diet deficient in, but otherwise adequate, growth and calcification on . . . . .
- Phosphorus and calcium, dietary, effect of, on assimilation of dietary fluorine . . . . .
- Phosphorus and calcium, storage of, and fetal growth, dependence of, on the parathyroid function and diet of pregnant rats . . . . .
- Plane of nutrition and environmental temperature, influence of, on relationship between basal metabolism and endogenous nitrogen metabolism subsequently determined . . . . .
- Plasma, blood, magnesium content of, effect of magnesium salts and various natural feeds upon. Magnesium studies in calves. II . . . . .
- Pregnancy, ascorbic acid content of cow's milk during . . . . .
- Pregnant rats, dependence of fetal growth and storage of calcium and phosphorus on the parathyroid function and diet of . . . . .
- Prenatal diet, influence of, on mother and child . . . . .
- Prevention of muscular dystrophy in the young, and reproduction in the female,  $\alpha$ -tocopherol requirement of the rat for . . . . .
- PRINGLE, EXMUR M. See Barott, Herbert G. . . . .
- Production, dietary, of cataracts in larval *Ambystoma tigrinum* . . . . .
- Production of high vitamin A milk by diet . . . . .

- Products, fish, riboflavin content of  
Products, milled, rye and its, thiamine,  
nicotinic acid, riboflavin and panto-  
thenic acid in ..... 425
- Protein feeding, influence of previous  
regimes of, on endogenous nitrogen  
metabolism of rats ..... 527
- Protein intake, influence of, upon  
growth, reproduction and longevity  
studied at different calcium levels ..... 383
- Protein, synthesis of, in paunch of  
ruminant, value of urea in. I. In  
maintenance ..... 327
- II. In growth ..... 167
- Proteins in cottonseed meal, effect of  
autoclaving on nutritive value of ..... 183
- Q**UARLES, ERNESTINE. See Snell,  
Esmond E. ..... 483
- R**ABBITS, minimum requirement of,  
for dl-a-tocopherol ..... 123
- Rat, albino, growth curve of, in relation  
to diet ..... 205
- Rat, a-tocopherol requirement of, for  
reproduction in female and preven-  
tion of muscular dystrophy in  
the young ..... 463
- Rat, carbohydrate storage and mobili-  
zation in ..... 255
- Rat caries and composition of rats'  
teeth, effect of fluoride on. Ob-  
servations on induced dental caries  
in rats. III ..... 391
- Rat caries, experimental, reduction in,  
by fluorine ..... 255
- Rat growth assay method for ribo-  
flavin, studies on ..... 345
- Rat, pathology of riboflavin deficiency  
in ..... 103
- Ration, seleniferous wheat, excretion of  
selenium by rats on ..... 541
- Rations, balanced, composed of com-  
binations of different feeds, utiliza-  
tion by calves or energy contained  
in ..... 511
- Rats and mice, differences in behavior  
of, towards deficiencies of certain  
members of the vitamin B complex ..... 439
- Rats, assimilation of fluorine by, from  
natural and synthetic cryoalite and  
from cryolite-sprayed fruits ..... 451
- Rats, endogenous nitrogen metabolism  
of, influence of previous regimes  
of protein feeding on ..... 383
- Rats fed a diet extremely low in zinc,  
histological studies of tissues of ..... 223
- Rats, growing, severe calcium deficiency  
in. III. Serum calcium of indi-  
vidual animals during develop-  
ment of calcium deficiency ..... 1
- Rats, inefficacy of hormones in nutri-  
tional achromotrichia of ..... 553
- Rats, observations on induced dental  
caries in. III. Effect of fluoride  
on rat caries and on composition  
of rats' teeth ..... 391
- Rats on a seleniferous wheat ration,  
excretion of selenium by ..... 103
- Rats, pregnant, dependence of fetal  
growth and storage of calcium and  
phosphorus on the parathyroid  
function and diet of ..... 25
- Rats, studies on nutritional achromot-  
richia in ..... 565
- Rats' teeth, composition of, effect of  
fluoride on rat caries and on.  
Observations on induced dental  
caries in rats. III ..... 391
- Rats, young, improved diets for nutri-  
tional and pathologic studies of  
choline deficiency in ..... 109
- Reduction in experimental rat caries  
by fluorine ..... 255
- Regimes, previous, of protein feeding,  
influence of, on endogenous nitro-  
gen metabolism of rats ..... 383
- Relative assimilation of fluorine from  
fluorine-bearing minerals and  
food (tea), and from water and  
food ..... 621
- Reproduction, growth and longevity  
studied at different calcium levels,  
influence of protein intake upon ..... 327
- Reproduction in female and preven-  
tion of muscular dystrophy in the  
young, a-tocopherol requirement of  
rat for ..... 463
- Requirement, minimum, of rabbits for  
dl-a-tocopherol ..... 415
- Requirements, dietary, for fertility and  
lactation ..... 491
- "Residue, indigestible," in whole wheat  
and white breads, apparent digesti-  
bility of carbohydrates, fats, and  
Review, editorial. The nutritional im-  
portance of choline ..... 589
- Riboflavin as a food factor in economy  
of food utilization, further observa-  
tions on ..... 295
- Riboflavin content of fish products ..... 425
- Riboflavin deficiency in dog, further  
observations of ..... 7
- Riboflavin deficiency in rat, pathology  
of ..... 345
- Riboflavin, pantothenic acid, thiamine  
and nicotinic acid in rye and its  
milled products ..... 527
- Riboflavin, studies on rat growth assay  
method for ..... 399
- R**ICHARDS, GRACE V. See Unna, Klaus
- R**OUTH, JOSEPH I. See French, Row-  
land B. ..... 383
- Ruminant, paunch of, value of urea  
in synthesis of protein in. I. In  
maintenance ..... 167
- II. In growth ..... 183
- R**USSELL, RAYMOND A., AND EDMUND  
S. NASSER. The effects of various  
vitamin supplements and of whole  
yeast on the digestion and absorp-  
tion of the carbohydrate of a com-  
plete diet ..... 287
- Rye and its milled products, thiamine,  
nicotinic acid, riboflavin and  
pantothenic acid in ..... 527
- S**ALMON, WILLIAM D. See Engel,  
Ruben W. ..... 109
- Salts, magnesium, and various natural  
feeds, effect of, upon magnesium  
content of blood plasma. Mag-  
nesium studies in calves. II ..... 609
- S**AMPSON, W. L. See Unna, Klaus
- S**ATTERFIELD, GEORGE H. See Holmes,  
Arthur D. ..... 267
- S**CHLUTZ, FREDERIC W. See Shukers,  
Carroll F. ..... 53
- S**CHUETTE, HENRY A. See Ihde, Aaron  
J. ..... 527
- S**COFT, W. A. See Ebbs, J. H. ..... 515
- S**EARLOCK, ROBERT E., DANIEL H.  
BASINSKI AND JOHN R. MURKIN. Apparent  
digestibility of carbo-  
hydrates, fats, and "indigestible  
residue" in whole wheat and white  
breads ..... 589
- Selenium, excretion of, by rats on a  
seleniferous wheat ration ..... 103

- Severe calcium deficiency in growing rats. III. Serum calcium of individual animals during development of calcium deficiency . . . . .
- SHAW, JAMES H., AND PAUL H. PHILLIPS. The pathology of riboflavin deficiency in the rat . . . . .
- SHERMAN, HENRY C. See Kao, Hsueh-Chung . . . . .
- SHERMAN, WILLIAM C. The effect of certain fats and unsaturated fatty acids upon the utilization of carotene . . . . .
- SHUKERS, CARROLL F., ELIZABETH M. KNOTT AND FREDERIC W. SCHLUZT. Magnesium balance studies with infants . . . . .
- SNELL, EDMOND E., AND ERNESTINE QUARLES. The effect of incubation on the vitamin content of eggs . . . . .
- SNIDER, GEORGE G. See Hoagland, Ralph . . . . .
- Soybeans, goitrogenicity of . . . . .
- Steam-rendered lard and hydrogenated cottonseed oil, nutritive properties of . . . . .
- Storage, carbohydrate, and mobilization in rat . . . . .
- Storage of calcium and phosphorus and fetal growth, dependence of, on the parathyroid function and diet of pregnant rats . . . . .
- STREET, HAROLD R. Studies on the rat growth assay method for riboflavin . . . . .
- STREET, HAROLD R., GEORGE R. COWGILL AND HARVEY M. ZIMMERMAN. Further observations of riboflavin deficiency in the dog . . . . .
- Studies on nutritional achromotrichia in rats . . . . .
- Studies on rat growth assay method for riboflavin . . . . .
- Supplements, other dietary, and certain organic compounds, effect of, on perosis . . . . .
- Supplements, various vitamin, and whole yeast, effects of, on digestion and absorption of the carbohydrate of a complete diet . . . . .
- SURE, BARNETT. Further observations on riboflavin as a food factor in economy of food utilization . . . . .
- SURE, BARNETT. Dietary requirements for fertility and lactation. XXVIII. The lactation-promoting properties of cystine when added to casein diets . . . . .
- SURE, BARNETT. Dietary requirements for fertility and lactation. XXIX. The existence of a new dietary factor essential for lactation . . . . .
- Survey, nutrition (North Carolina), dark adaptometer and blood vitamin A measurements in . . . . .
- SWANK, ROY L., AND OTTO A. BESSEY. Characteristic symptoms and their pathogenesis. III. Avian thiamine deficiency . . . . .
- Synthesis of protein in paunch of ruminant, value of urea in. I. In maintenance . . . . .
- II. In growth . . . . .
- Synthetic and natural cryolite and cryolite-sprayed fruits, assimilation of fluorine by rats from . . . . .
- TEA (food), and fluorine-bearing minerals, and water and food. relative assimilation of fluorine from . . . . .
- Teeth, rats', composition of, effect of fluoride on rat caries and on. Observations on induced dental caries in rats. III . . . . .
- Temperature, energy and gaseous metabolism of hen as affected by Temperature, environmental, and plane of nutrition, influence of, on relationship between basal metabolism and endogenous nitrogen metabolism subsequently determined . . . . .
- Thiamine deficiency, avian. Characteristic symptoms and their pathogenesis. III . . . . .
- Thiamine, nicotinic acid, riboflavin and pantothenic acid in rye and its milled products . . . . .
- Tigrinum, larval Amblystoma, dietary production of cataracts in . . . . .
- TISDALE, F. F. See Ebbs, J. H. . . . .
- Tissues of normal and blacktongue dogs, nicotinic acid and coenzyme content of . . . . .
- Tissues of rats fed a diet extremely low in zinc, histological studies of . . . . .
- Tocopherol (*α*), requirement of the rat for reproduction in the female and prevention of muscular dystrophy in the young . . . . .
- Tocopherol (*dL-α*), minimum requirement of rabbits for . . . . .
- TREICHLER, RAY, AND HAROLD H. MITCHELL. The influence of plane of nutrition and of environmental temperature on the relationship between basal metabolism and endogenous nitrogen metabolism subsequently determined . . . . .
- TRIPP, FRANCIS. See Holmes, Arthur D. . . . .
- UNNA, KLAUS. See Muschett, Charles W. . . . .
- UNNA, KLAUS, GRACE V. RICHARDS AND W. L. SAMPSON. Studies on nutritional achromotrichia in rats . . . . .
- Unsaturated fatty acids and fats, effect of certain, upon utilization of carotene . . . . .
- Urea, value of, in synthesis of protein in paunch of ruminant. I. In maintenance . . . . .
- II. In growth . . . . .
- Utilization by calves of the energy contained in balanced rations composed of combinations of different feeds . . . . .
- Utilization, food, further observations on riboflavin as a food factor in economy of . . . . .
- Utilization of calcium of cauliflower and broccoli . . . . .
- Utilization of carotene, effect of certain fats and unsaturated fatty acids upon . . . . .
- VALUE, biological, and digestibility, of whole wheat breads as compared with white bread . . . . .
- Value, nutritive, of proteins in cottonseed meal, effect of autoclaving on . . . . .
- Value of urea in synthesis of protein in the paunch of the ruminant. I. In maintenance . . . . .
- II. In growth . . . . .

- Vitamin A measurements, blood, and dark adaptometer, in a North Carolina nutrition survey ..... 597
- Vitamin A milk, high, by diet, production of ..... 303
- Vitamin B complex and fat metabolism ..... 359
- Vitamin B complex, deficiencies of certain members of, differences in behavior of rats and mice towards ..... 489
- Vitamin content of eggs, effect of incubation on ..... 483
- Vitamin supplements (various), and whole yeast, effects of, on digestion and absorption of the carbohydrate of a complete diet ..... 287
- WAISMAN, HARRY A.** See McIntire, J. M. ....
- Water and food, and fluorine-bearing minerals and food (tea), relative assimilation of fluorine from ..... 287
- Wheat breads, whole, as compared with white bread, digestibility and biological value of ..... 589
- Wheat ration, seleniferous, excretion of selenium by rats on ..... 103
- Wheat, whole, and white breads, apparent digestibility of carbohydrates, fats, and "indigestible residue" in ..... 287
- Whole yeast, and various vitamin supplements, effects of, on digestion and absorption of the carbohydrate of a complete diet ..... 43
- ZIMMERMAN, HARRY M.** See Street, Harold E. ..... 573
- Zinc, histological studies of tissues of rats fed diet extremely low in ..... 223
- ZUCKER, LOIS.** See Zucker, Theodore F. ..... 123
- ZUCKER, THEODORE F., LILIAN HALL MARGARET YOUNG AND LOIS ZUCKER.** The growth curve of the albino rat in relation to diet ... 123
- ZUCKER, THEODORE F., LILIAN HALL AND MARGARET YOUNG.** Growth and calcification on a diet deficient in phosphate but otherwise adequate ..... 139



A. R. L. 75-

**IMPERIAL AGRICULTURAL RESEARCH  
INSTITUTE LIBRARY  
NEW DELHI.**